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RATES OF SODIUM TURNOVER IN NORMAL SUBJECTS AND IN PATIENTS WITH CONGESTIVE HEART FAILURE

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THERE is a need for a study of the rate with which sodium escapes from the blood into the tissue spaces and urine of man, especially because of the present interest in the role of sodium in congestive heart failure and other types of edema. Although studies of sodium metabolism have been conducted in man by means of radiosodium,[1-4] these have not included a detailed tracing of the variations in sodium concentration in the blood, urine, and sweat. The rates of diffusion of sodium through the vascular wall have been determined for other animals[5, 6] but not for man. Furthermore, tracer studies in congestive heart failure with the use of radiosodium have not been conducted previously. These studies are concerned with such problems.

METHODS

The subjects were all patients at the Charity Hospital. For purposes of discussion, the subjects were divided into three groups: normal subjects, those with congestive heart failure, and miscellaneous subjects. Certain detailed information concerning them is given in Table I. The ten normal subjects were patients who had entered the hospital for observation but were clinically well when studied. The patients with congestive heart failure suffered with chronic congestive heart failure of Functional Class IV and were bedridden. All were under treatment and were improving.

Because of the 14.8-hour half life of the radioactive tracer, Na^{24}, and the uncertainty of delivery from the cyclotron laboratories it was not possible to keep patients without treatment in preparation for the experiments. The influence of various therapeutic measures and other details concerning congestive heart failure were evaluated in studies conducted with the use of the long-life sodium, Na^{22}.[7, 8] The results of those studies will appear from time to time elsewhere in a series of papers, one report[7] having been published.

All subjects were at least three hours postprandial. They were taken to the laboratory where ureteral catheters were placed‡ into the pelves of each kidney of the normal subjects. The first subjects studied received no drugs in preparation for the cystoscopy while the later ones received morphine

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TABLE I. CLINICAL DATA CONCERNING THE NEGRO SUBJECTS STUDIED*

SUBJECT	SEX	AGE	WT.	DIAGNOSIS	TREATMENT	Na^{23} IN URINE (MEQ./L.) MEAN	MAX.	MIN.	Na^{23} SPACE TIME OBSERVED (C.C.) 60 MIN.	90 MIN.
					Normal Subjects					
1	F	37	152	Rheumatoid arthritis	None	123	128	119	12180	-----
2	F	32	119	Rheumatoid arthritis	None	176 212	182 219	179 (lt.) 203 (rt.)	16598	-----
3	F	24	94	Pneumonitis	Vitamins	116 119	120 123	112 (rt.) 115 (lt.)	8587	-----
4	F	17	108	Syphilis	None	100	103	84	11689	11891
5	F	24	118	Anxiety neurosis	Na salicylates	144	149	139	11786	-----
6	F	48	110	Syphilis	None	88	91	85 (lt.)	11534	12508
7	F	23	146	Normal	None	185	192	187	9132	-----
8	F	32	100	Normal	None	209 194	216 201	202 (rt.) 187 (lt.)	6124	-----
9	F	19	92	Chronic bronchitis	None	216 209	223 216	208 (rt.) 202 (lt.)	10301	11198
10	F	34	130	Anxiety neurosis	None	263	272	254	8961	-----
Mean						163 157 (lt.) 188 (rt.)	171 163 (lt.) 195 (rt.)	157 154 (lt.) 181 (rt.)	10689	11866
Maximum							272	254	16598	12508
Minimum							91	84	6124	1198
					Subjects With Congestive Heart Failure					
1	F	66	121	Arteriosclerotic heart disease, congestive heart failure, syphilis, auricular fibrillation	Digitalis, salt-free diet	62	64	51	12747	16556
2	F	65	159	Hypertensive cardiovascular disease, congestive heart failure	NH_4Cl, salt-free diet, digitalis	132	13.7	12.8	12461	13559
3	F	36	129	Hypertensive cardiovascular disease, congestive heart failure	Salt-free diet, NH_4Cl, digitalis	162	16.7	15.6	17197	21186
5	F	46	125	Hypertensive cardiovascular disease, congestive heart failure	Digitalis, salt-free diet	52	53	50	10315	10220
6	F	53	122	Hypertensive cardiovascular disease, congestive heart failure	Digitalis, salt-free diet, Hg (occasionally)	119	123	115	17668	17762
7	M	32	137	Hypertensive cardiovascular disease, congestive heart failure	Salt-free diet, NH_4Cl, digitalis				10163	9259
8	F	59	181	Arteriosclerotic heart disease, congestive heart failure	Salt-free diet, NH_4Cl, digitalis	136	141	132	28624	-----
9	F	61	148	Arteriosclerotic heart disease, congestive heart failure		40	41	38	12895	16461

TABLE I—CONT'D

SUBJECT	SEX	AGE	WT.	DIAGNOSIS	TREATMENT	Na^{23} IN URINE (MEQ./L.) MEAN	MAX.	MIN.	Na^{23} SPACE TIME OBSERVED (C.C.) 60 MIN.	90 MIN.
10	F	67	140	Arteriosclerotic heart disease, congestive heart failure	Salt-free diet, digitalis, salyrgan, NH_4Cl	253	261	239	14674	16736
11	M	62	166	Hypertensive cardiovascular disease, congestive heart failure	NH_4Cl, digitalis, salt-free diet	100	103	98	9029	9970
Mean						87.9	90.7	83.5	14577	14634
Maximum						13.2	26.1	23.9	28624	21186
Minimum						25.3	13.7	12.8	9029	9259
Subjects With Miscellaneous Disease States										
1	F	41	151	Hypertensive cardiovascular disease, congestive heart failure, ovarian malignancy	NH_4Cl, digitoxin salt-free diet	31	32	30	25157	23474
2	F	27	110	Chronic active hemorrhagic nephritis	Low-salt diet				14535	15662
3	F	37	134	Sarcoidosis	Symptomatic	52	53	50	10899	10701
4	F	42	141	Hypertensive cardiovascular disease, pylonephritis vs. chronic active hemorrhagic nephritis	No diuretic or edema therapy	90	93	87	20833	20618
5	F	50	109	Phlebothrombosis of deep leg veins	No special therapy	25	26	24	10315	11561
6	F	36	173	Cold allergy, anxiety neurosis	None for edema, kidney	132	137	128 (rt.)	10096	1154
7	M	15	110	Chronic adhesive pericarditis	NH_4Cl, salt-free diet, paracentesis	26.5	27.4	25.6		
Mean						44.9	46.3	43.3	15306	15528
Maximum						90	93	87	25157	23474
Minimum						25	26	24	10096	10701

*The concentration of the sodium (Na^{23}) in the urine was determined on the basis of a normal blood sodium concentration. The "sodium space" was calculated on the basis of a state of equilibrium at the times indicated. The normal subjects were clinically well when studied; the diagnoses indicated were those made at the time of admission to the Charity Hospital for study.

sulfate, 15 mg., and atropine sulfate, 0.3 mg. subcutaneously thirty minutes before the cystoscopy. The results showed evidence of influence by the drugs used in preparation of the subjects for cystoscopy. The patients with congestive heart failure and two normal subjects had a small catheter passed only into the bladder. The patients with congestive heart failure were too ill to permit a cystoscopy to place catheters into the renal pelves. Bladder urine was about as informative as the pelvic urine.

After catheterization, a sample of urine was collected from each catheter and a sample of blood was collected simultaneously from the antecubital vein of one arm through a heparinized needle. The radioactive tracer, Na^{24} (0.5 to 2 c.c. of a neutral and hypotonic aqueous solution of NaCl), with an activity

of 20,000,000 counts per minute (approximately 0.1 mc. or 0.04 r) was injected quickly into the antecubital vein of the opposite arm. Two cubic centimeter samples of blood were collected for serum studies as rapidly as possible, every five to ten seconds unless the needle became obstructed and necessitated a greater interval between collections. This rate of collection was followed for the first three minutes after the injection of the Na^{24}. The interval between collections was increased to thirty seconds for the next five minutes, increased again to every sixty or 120 seconds for the next ten minutes, and finally, to every three, five, or ten minutes for the remainder of the study, which lasted from sixty to one hundred eighty minutes. The urine samples were collected from each catheter in separate vials. For the first three minutes the interval between collections of the urine samples varied with the rate of urine flow, that is, long enough for four drops of urine to be collected. This usually required five to fifteen seconds. For the next five minutes collections were made at thirty-second intervals, etc., as for the blood samples. Attempts were made to collect the blood and urine samples at the same time in order to permit satisfactory comparisons. The volume of the urine samples was measured to determine the sodium (Na^{24}) clearances from collection to collection. The urine samples collected from the pelvis of each kidney were studied separately.

In several experiments samples of edema fluid were collected simultaneously through a puncture wound made through the skin near the ankle with an 18-gauge needle. The rates of collection approximated those for the blood and urine. Simultaneous collections of successive samples of ascitic and pleural fluid in subjects with congestive heart failure were made to study the change in concentration of Na^{24}.

One normal subject was placed in a hot and humid room (temperature, 48° C.; relative humidity, 45 per cent) free from drafts. After sweating had become profuse the procedure described above was followed except that the urine was collected at less frequent intervals while the blood and sweat samples were collected at the rapid rates stated previously. The sweat was collected by allowing the beads of sweat to roll into the opening of a tall narrow-mouthed vial which was promptly closed with a bakelite screw cap. Each sample of sweat consisted of about 0.1 cubic centimeter. These collections were continued for about thirty minutes, that is, until the subject and observers began to experience marked discomfort from the hot and humid environment.

The concentration of Na^{24} in all samples was expressed as counts per cubic centimeter per minute. Geiger-Muller counters* were used to determine radioactivities of the injections and the samples. Corrections were made for background counts and decay. The entire counting method had an over-all error of less than 3 per cent. All dosages of Na^{24} were reduced to the common value of 20,000,000 counts per minute per 100 pounds of weight.

RESULTS

Normal Subjects.—The results for the normal subjects are summarized in Figs. 1 to 6.

*Technical Associates, Glendale, Calif.

Blood: It can be seen from Fig. 1 that the concentration of the Na^{24} rose rapidly in the *blood serum*, fluctuating in concentration for the first five to fifteen minutes and then becoming relatively constant for the remainder of the period of observation. The concentration of Na^{24} did not rise to levels as high as would be expected if all the Na^{24} remained in the blood stream. There was a tendency for the concentration of Na^{24} in the serum of most of the subjects to rise for the first ten to twenty minutes to levels higher than those reached during the final periods of the study and to show marked variations from subject to subject. There was a gradual decline in the serum concentration of Na^{24} in all subjects throughout the entire period of study even after a fairly stable state had been reached. The Na^{24} did not appear in the antecubital vein of the sampling arm until nineteen to twenty-three seconds after the radiosodium had been injected into the antecubital vein of the other arm, that is, by a time equal to the circulation time.

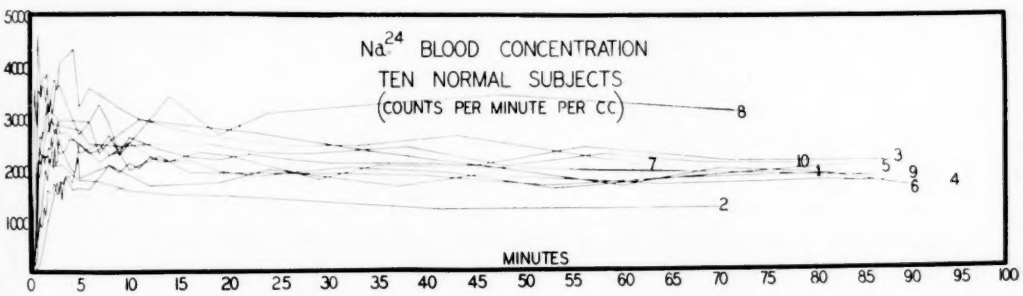

Fig. 1.—Variations in serum concentration of Na^{24} in ten normal subjects. There are considerable individual variations, especially during the first two to three minutes when the mixing of the injected radiosodium is occurring.

Because of the marked variations in concentration of Na^{24} in the serum of the subjects and because of the small number of subjects studied, it was impossible to obtain a composite tracing which properly depicted the rate of diffusion of sodium from the vascular bed (Fig. 1). Therefore, the data on a subject (Subject 6) were selected for analysis in the manner described by Gellhorn and co-workers;[5] all diffusion curves were drawn by inspection. These authors showed that the vascular bed of dogs could be considered to be composed of two separate areas through which the sodium ion diffuses at different rates. Area A_1 consists of an area through which the sodium diffuses at a very rapid rate, while A_2 represents another area through which the rate of diffusion is relatively slow. The same types of areas were observed in these studies in the normal and diseased man. Fig. 2 can be seen to represent a typical double exponential curve for the blood concentration. There was a *rapid rate* of escape of Na^{24} from the vascular bed and a *slow one*. The general expression obtained by Gellhorn and co-workers[5] for dogs, and found to apply to man in many of the subjects of this study, is

$$c_p - c_{eq} = a_1\epsilon^{b_1 t} + a_2\epsilon^{b_2 t} \qquad (1)$$

where c_p is plasma concentration of Na^{24} at time, t, c_{eq} is plasma concentra-

tion at equilibrium, b_1 and b_2 are rates of diffusion from the vascular bed, and a_1 and a_2 are coefficients which depend upon the rates of movement and distribution of Na^{23} through areas A_1 and A_2 in the vascular bed. Since the diffusion of Na^{24} is determined by the diffusion of Na^{23}, the exponents b_1 and b_2 are expressions of the rates of diffusion of the total plasma sodium (Na^{23}) out of the vascular bed. Therefore, it is possible to consider the sodium of the plasma as being divided into three parts: one represented by a_1, which diffuses outward rapidly through area A_1; a second represented by a_2, which diffuses outward at a relatively slow rate through area A_2; and a third represented by c_{eq}, which remains in the plasma.

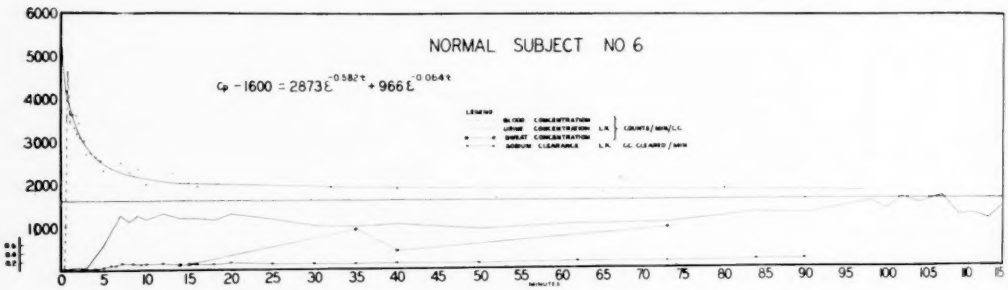

Fig. 2.—Variations in serum, urine, and sweat concentrations of Na^{24} and the Na^{24} clearance in normal Subject 6. The curve of blood concentration is a double exponential curve representing sodium diffusion, the equation of which is shown. *L.K.*, Left kidney.

To determine the rate of loss by diffusion of the total plasma sodium, the equation

$$\frac{-a_1b_1 - a_2b_2}{c_o} = R_p$$

is employed, where c_o is the extrapolated initial count per minute per cubic centimeter of the plasma.

The expression for radioactive sodium concentration in the plasma for Subject 6 (normal) was

$$c_p - 1600 = 2873\epsilon^{-0.582\ t} + 966\epsilon^{-0.0644\ t}. \qquad (2)$$

Thus 58 per cent of the rapidly diffusing sodium left the plasma per minute, and 6.4 per cent of the slowly diffusing sodium left the plasma per minute. Considering the sizes of these sodium compartments in the plasma, an average of 32 per cent of the total sodium in the plasma diffused out of the vascular bed per minute.

Furthermore, Merrell and associates[6] have shown that from the same data the rate of diffusion of sodium from the extravascular fluid* back into the plasma at equilibrium could be determined by the equation

$$R_e = R_p \frac{(1-q)}{q}$$

*The term extravascular sodium employed here probably is concerned predominantly with interstitial fluid sodium because of the relatively short duration of the studies. For that reason interstitial and extravascular sodium is considered essentially synonymous in these studies.

where R_e = rate at which the extravascular sodium returns by diffusion into the plasma, R_p = rate of loss by diffusion of the total plasma sodium in the vascular bed, and $_q$ = total extravascular sodium. The values obtained for Subject 6 are shown in Table II. It is noted that about 32 per cent of the total sodium in the plasma diffused into the interstitial fluid per minute and that 13 per cent of the total sodium in the interstitial fluid returned by diffusion to the plasma per minute.

TABLE II. PROPORTION OF SODIUM DIFFUSION IN FOUR HUMAN SUBJECTS

	NORMAL SUBJECT	CONGESTIVE HEART FAILURE SUBJECT		MISCELLANEOUS SUBJECT
	6	3	1	5
Proportion of total sodium in interstitial fluid	0.70	0.89	0.90	0.65
Proportion of total plasma sodium diffusing out of vascular bed per minute	0.32	0.63	0.54	0.35
Proportion of total interstitial fluid sodium diffusing into the vascular bed per minute	0.13	0.08	0.06	0.18

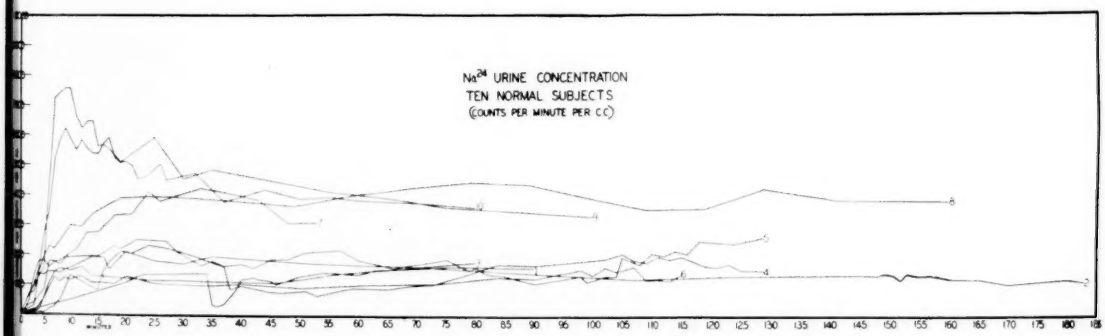

Fig. 3.—Variations in urine concentrations of Na^{24} in ten normal subjects.

Urine: The concentration of radioactive sodium in the urine exceeded that in the blood serum in three subjects (Fig. 3). In most cases the blood and urine concentrations were essentially equal, although the urine concentration was definitely lower in Subject 6. The urine concentration varied much more than did the blood concentration after the initial few minutes following the injection.

Sodium Clearance: The sodium clearances for five normal subjects are shown in Figs. 4, 5, and 6. Na^{24} clearances were employed in these analyses since they integrated volume of urine flow and blood and urine concentrations. The exact significance of these values for a substance like sodium is not known. They do make certain comparisons feasible. These values were calculated in a manner similar to that used for the urea clearance.

The equation for the calculation is

$$Na^{24}\ \text{clearance} = \frac{Na_u^{24}\ U_v}{Na_p^{24}} \qquad (3)$$

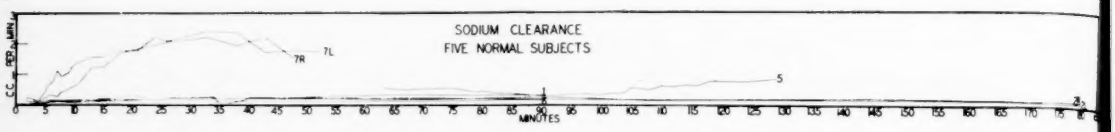

Fig. 4.—Variations in Na^{24} clearance in five normal subjects. Since the movement of Na^{24} around the body is determined by the movement of Na^{23}, these curves represent rates of Na^{23} clearance once equilibrium has been reached.

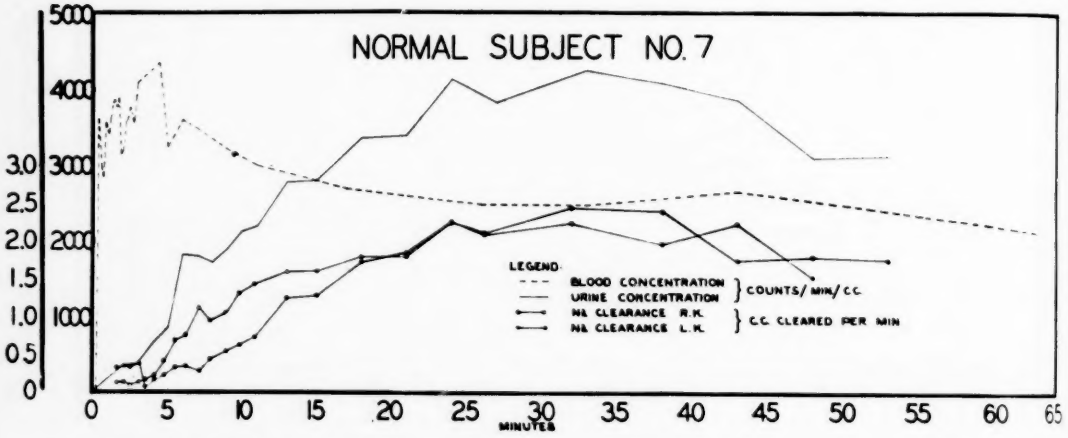

Fig. 5.—Variations in serum and urine concentration (left kidney) of Na^{24}, and Na^{24} clearance for both kidneys separately in one subject. Note the marked variations in serum concentration during the first three minutes. The serum concentration then settled down to a diffusion type of curve as shown for normal Subject 6 (Fig. 2). The mixing phenomenon was relatively poor for the first three minutes in Subject 7, but early and adequate for Subject 6. Such chance variations are to be expected. Note the concordant and only occasional discordant relationship of the Na^{24} clearances in the urines of the right and left kidneys.

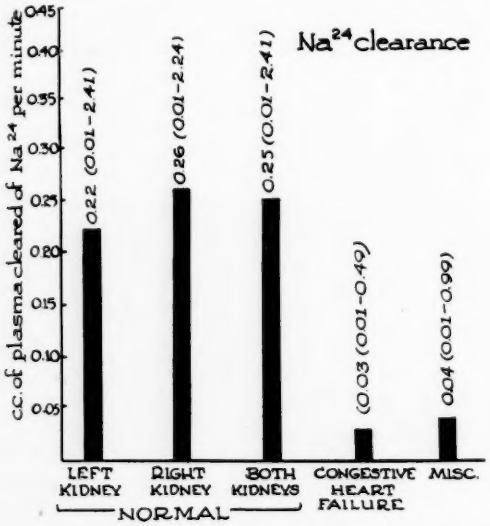

Fig. 6.—The average and extreme (within parentheses) values for Na^{24} clearance in the three groups of subjects. It is necessary to remember that the patients with congestive heart failure were on a slow sodium diet.

where Na_u^{24} = counts per cubic centimeter of urine, Na_b^{24} = counts per cubic centimeter of blood, and U_v = volume of urine flow per minute.

Except for Subject 7 the rates of Na^{24} clearance were fairly constant and of essentially the same magnitude. They tended to average 0.22 c.c. of blood cleared per minute per kidney but were high for Subject 7, reaching a level of about 2.4 c.c. of blood cleared of Na^{24} per minute for both kidneys. Figs. 4, 5, and 6 show the quantitative and qualitative variations in the clearances for both kidneys observed separately and simultaneously.

Comment: The levels of Na^{24} concentration in the blood serum should have reached values of about 10,000 counts per minute per cubic centimeter if all of the injected materials had remained in the blood. Instead, the concentrations actually reached averaged about 2,500 counts per minute per cubic centimeter, with the maximum being 4,600. The average curve for the normal subject shows a tendency for the concentration of Na^{24} in the blood to reach the final concentration level rapidly and not to exceed it appreciably. This indicates the very rapid rate of diffusion of sodium from the blood. In fact, the diffusion is so rapid that the circulation of the blood is too slow to permit adequate mixing before a considerable amount of diffusion occurs. The relatively marked variations in Na^{24} concentration in the blood observed during the first few minutes of the study are due in a large part to inadequate mixing. Adequate mixing does not appear to be completed in some subjects before ten minutes have elapsed (Fig. 1). As stated previously, the marked variations in the rates of mixing of the blood made it impossible to determine the average diffusion rates for the normal subjects as a group. From the analysis of the data of Subject 6 it was found that sodium diffused across the vascular bed at two separate rates. This diffusion outward resulted in a loss per minute of about 0.6 of the rapidly diffusing and of about 0.06 of the slowly diffusing plasma sodium. The average rate of diffusion of sodium from the vascular bed was 32 per cent of the total plasma sodium per minute. This is equal to about 4,000 Gm. of sodium per day for a man weighing 70 kilograms. These results are remarkably similar to those observed in dogs by Gellhorn and associates.[5] It is interesting to note that if the Cl ion tends to follow the Na ion during its diffusion, this would entail a diffusion of about 10,000 Gm. of NaCl out of the vascular bed of the average man per day. The amount of sodium returning is, of course, the same. Thus there is a diffusion of about fifty pounds of NaCl back and forth through the capillary wall per day in a man weighing 70 kilograms.

It is generally agreed that the rapid diffusion of sodium is into the interstitial fluid.[5] The slower diffusion must take place into bone,[2, 9] cerebrospinal fluid,[10] chambers of the eye,[10] and other structures of the body which allow relatively slow rates of sodium exchange. It is also important to know the rate of diffusion of water across the vascular bed. Merrell and co-workers[6] observed that 1.46 of the plasma water diffused out into the tissues of the guinea pig per minute. Since comparable values were obtained for the rates of diffusion of sodium in guinea pigs, dogs, and man, it is likely that the rates of diffusion of water should be similar. If the same rates are applied for man as were

found in the guinea pig, then about 6,300 liters of water per day diffuses out of the vascular bed of a man weighing 70 kilograms. It is obvious from the diffusion rates for sodium and water that water diffuses more rapidly. Not only has this been observed for the body as a whole, but for isolated tissues as well. Kinsey and associates[10, 11] found this to be true for the vitreous body and aqueous humor of the eye of the rabbit. It has also been noted for the cerebrospinal fluid.[12]

Although the Na^{24} concentration in the blood serum reached a level free from marked fluctuations within a few minutes, the concentrations progressively decreased and did not reach a state of equilibrium by the end of two hours, even though corrections were made for loss of Na^{24} in the urine. These findings, therefore, indicate that the Na^{24} rapidly reaches equilibrium with the interstitial fluid. This represents the greatest portion of the Na^{24} injected. Then for several hours there is a gradual passage of the Na^{24} into bone, spinal fluid, vitreous body, aqueous humor, and similar sodium compartments.[9-11] Therefore, studies which require a complete state of equilibrium with radiosodium for all structures of the body must be conducted several hours after the injection of the tracer. The measurement of the sodium compartment of the body cannot be measured properly until the tracer has reached a state of equilibrium for all structures of the body. For that reason any estimate of the sodium compartment with the use of Na^{24} in the studies reported here is subject to error and therefore has limited significance.

The urine concentration of Na^{24} varied considerably from subject to subject. These differences must be related, in part, to the sodium intake preceding the experiments. The cause of the delay in the appearance of the Na^{24} in the urine is unknown. It must be due partly to the time required for the urine to flow from the glomerulus to the outlet of the catheter. However, this requires only about thirty seconds. The delay might be due in part to diffusion from the blood into Bowman's capsule, but the rapid appearance of Na^{24} in the sweat and the rapid diffusion into the interstitial fluid tends to eliminate such a delay as an important factor. The effect of pooling of urine in the pelves of the kidneys could not be evaluated.

Although the data are not shown for both kidneys separately, for all subjects the rates of excretion of Na^{24} were essentially the same for both kidneys. Although there were periods when the rates of excretion and Na^{24} concentration showed discordant relationships, the behavior was so similar as to make it unnecessary to report the data for both kidneys separately. Subject 7 (Fig. 5) exemplified the individual kidney relationships. Since the Na^{24} clearances are reported for only one kidney, the total clearances are essentially twice that shown for one kidney.

Although the concentration of Na^{24} in the urine varied quite markedly (Fig. 3), the rate of Na^{24} clearance was fairly constant. This indicates that when the volume of urine flow was great, the concentration of Na^{24} in the urine was proportionately low and vice versa.

Subjects With Congestive Heart Failure.—The results obtained in the subjects with congestive heart failure are summarized in Figs. 6 to 11.

Blood: There was a tendency for the concentration of Na^{24} in the blood serum to reach a higher level in the subjects with congestive heart failure than in the normal subjects (Fig. 7). Marked fluctuations were noted for the first ten to twenty minutes; then a stable level was reached. The injected Na^{24}

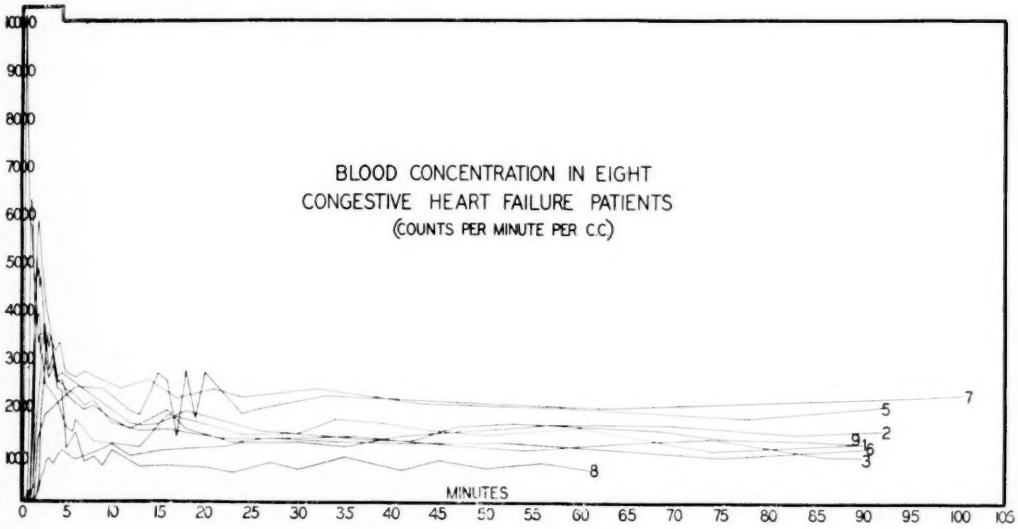

Fig. 7.—Variations in serum concentration of Na^{24} in eight patients with congestive heart failure. If the presence of a diffusion type of sodium curve indicates adequate and early mixing of the injected radiosodium, then mixing appears to have developed more quickly in these persons than in the normal ones.

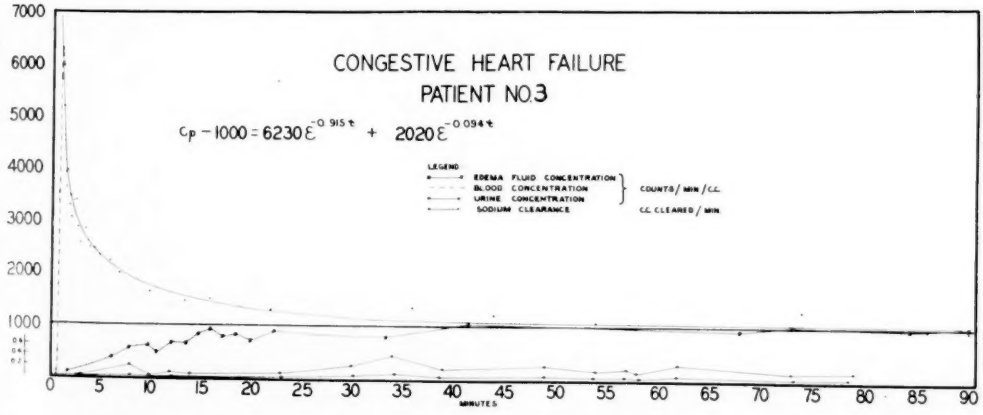

Fig. 8.—Variations in serum, urine, and edema fluid concentration and Na^{24} clearance in Patient 3 with chronic congestive heart failure. The serum concentration curve is a typical double exponential diffusion curve, the equation of which is shown. Note the fairly rapid increase in concentration of Na^{24} in the edema fluid to a level equal to that in the serum.

appeared in the antecubital vein of the sampling arm a few seconds later than in the normal subjects. Like the normal subjects, the patients with congestive heart failure showed such wide variations in the serum concentrations of Na^{24} that it was impossible to calculate a satisfactory average diffusion pattern for the Na^{24}. Some individuals, however, presented typical diffusion curves with

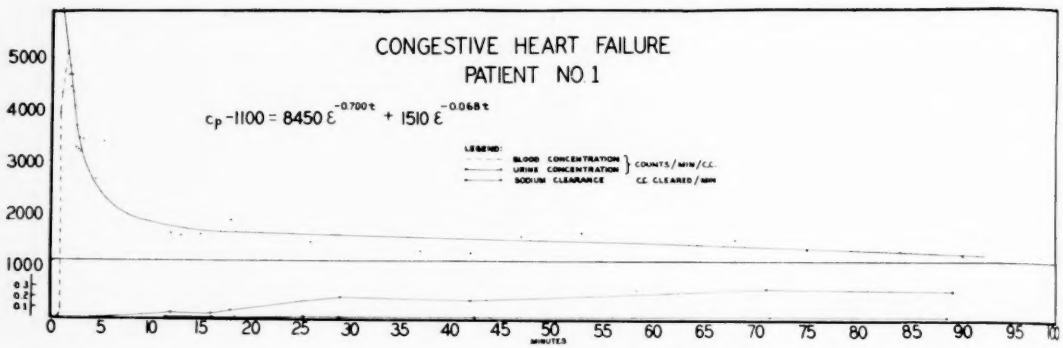

Fig. 9.—Variations in serum and urine concentration of Na^{24} and Na^{24} clearance in Subject 1 with congestive heart failure. The equation for the sodium diffusion curve is shown.

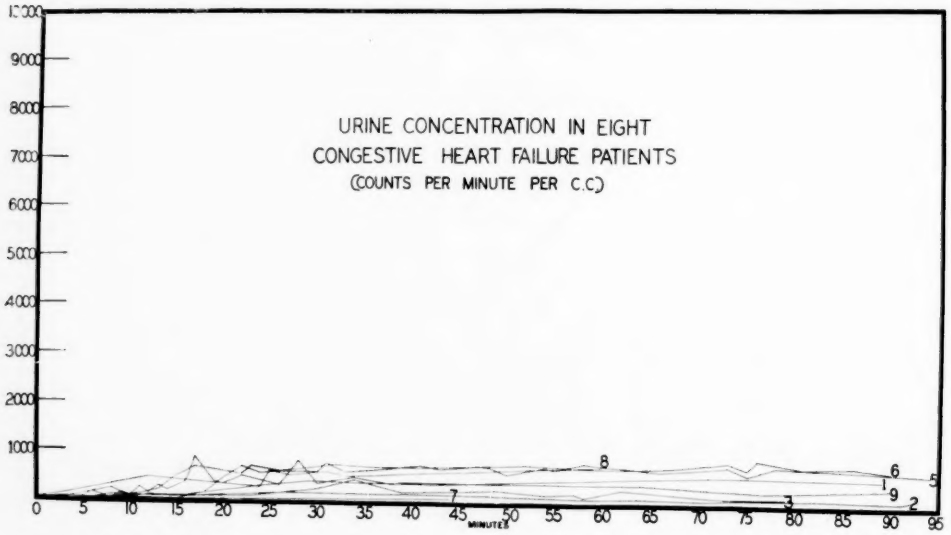

Fig. 10.—Variations in urine concentration of Na^{24} in eight patients with congestive heart failure and a low sodium diet. Compare these values with those for the normal (Fig. 3).

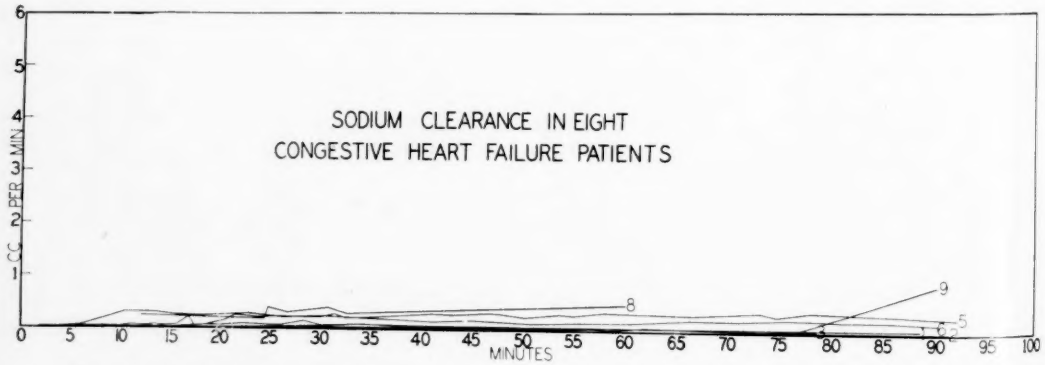

Fig. 11.—Variations in Na^{24} clearance in eight patients with congestive heart failure and a low sodium diet. Compare these values with those for the normal (Figs. 4 and 6).

both rapid and slow rates of diffusion of sodium from the blood plasma (Fig. 8). The equation for the diffusion curve of Fig. 8 was found to be:

$$c_p - 1000 = 6230\epsilon^{-0.915\ t} + 2015\epsilon^{-0.094\ t.} \quad (4)$$

This indicates that 0.915 of the plasma sodium which diffuses at the rapid rate escaped from the vascular bed into the interstitial spaces per minute, and 0.094 of the plasma sodium which diffuses at the slow rate escaped from the vascular bed per minute. Because of the rapid rate of diffusion in Subject 3, another patient (Subject 1) with congestive heart failure was similarly studied (Fig. 9). The curve for this patient had the following equation:

$$c_p - 1100 = 8450\epsilon^{-0.700\ t} + 1510\epsilon^{-0.068\ t.} \quad (5)$$

The diffusion rates in this subject were about the same as for the normal subject, although still more rapid.

From Table II it can be seen that 54 and 63 per cent of the total plasma sodium diffused out of the vascular bed per minute as average rates in Subjects 1 and 3, respectively. Simultaneously 6 and 8 per cent of the total interstitial fluid sodium diffused back into the plasma per minute in the respective patients.

Urine: The urine was collected from the bladder and not directly from the renal pelves as in the normal subjects. The results are summarized by Figs. 8, 9, and 10. The Na^{24} did not appear in the urine until four to five minutes after injection except in one patient (Fig. 10). The concentration of Na^{24} rose more slowly and the concentration levels reached were much lower than in the normal subjects. Considerable fluctuations were observed during the initial twenty to thirty minutes of study, followed by a stable level with minor variations of essentially the same degree as those encountered in the normal subjects.

Sodium Clearance: The sodium clearances of the patients with congestive heart failure with therapy are summarized in Figs. 6, 8, 9, and 11. The rates by which the blood was cleared of sodium were considerably lower than those observed for the normal subjects. These clearance values for both kidneys were about 0.03 c.c. per minute, the quantitative and qualitative variations being shown in Fig. 11. The clearance values approached zero in some subjects (Subjects 1, 2, and 3).

Edema Fluid: It was possible to follow the appearance of Na^{24} in the edema fluid of most of the patients with congestive heart failure. The results of the study of the edema fluid of Subject 3 are shown in Fig. 8.

Comment: The rapid and slow rates of diffusion of sodium from the vascular bed of the patients with congestive heart failure were higher than those observed in the normal subject. Obviously, it is not possible to conclude that such a difference is characteristic of congestive heart failure. Too few subjects were studied and too many factors are concerned with the escape of the sodium ion into the tissues for one to conclude that a greater rate of transcapillary diffusion is at work in all patients with congestive heart failure. The problem of mixing of the blood and the general inefficiency of the circula-

tion are important in congestive heart failure. Furthermore, it is necessary to bear in mind that all of the patients with congestive heart failure were receiving therapy. One aspect of the therapy (ammonium chloride and mercurial diuretics) was intended to make the kidneys excrete more sodium, while another phase of the therapy (dietary restriction of sodium) resulted in a decreased rate of renal excretion of sodium. The influence of these factors on the data obtained is unknown, but they were most probably great enough to make it impossible to evaluate the role of uncomplicated congestive heart failure on transcapillary diffusion of sodium. Gellhorn and associates[5] found the rates of transcapillary diffusion in dogs in shock, with and without treatment, were 50 per cent of those of normal dogs. Of course, the disturbances in hemodynamics are quite different in shock and in chronic congestive heart failure without shock.

Nevertheless, the more rapid rate of diffusion of sodium across the vascular wall in congestive heart failure than in the normal condition can be explained by the fact that there is a greater quantity of total amount of extracellular fluid and, therefore, of sodium in the edematous patients. Inspection of Table II shows that the part of the total extracellular fluid sodium of the patient with congestive failure diffusing back into the vascular bed per minute is about 50 per cent of that of the normal. The total quantity of sodium concerned, however, is greater than normal. Since the same quantity of sodium returns to the circulation as leaves it per unit time and since the increase in blood volume is not proportional to the increase in volume of the interstitial fluid, it is necessary that the plasma sodium diffuse more rapidly than in the normal subject in order to maintain a state of equilibrium. Thus the sodium of the interstitial fluid of the normal subject is turned over twice as rapidly as in the patients with congestive heart failure, whereas the sodium of the plasma of the latter patients is turned over twice as rapidly as that of the normal.

The rise in the concentration of Na^{24} in the edema fluid (Fig. 8) is an index of the rate of diffusion of normal sodium, Na^{23}, into edema fluid from the vascular bed. This rate is relatively high when the quantity of the edema fluid is considered. The concentration of the Na^{24} in the edema fluid reached that in the plasma, remaining near the plasma level thereafter. The duration of the rising periods of the Na^{24} concentration in the edema fluid is further support of the relatively long period of time required for a state of sodium equilibrium to be reached when tracer studies are done. This is particularly true when certain abnormal states are under observation.

Miscellaneous Subjects.—

Blood and Urine Concentrations and Sodium Clearance: The results are summarized in Figs. 12 to 15. The nature of the variations in Na^{24} concentration in the blood and urine and Na^{24} clearances was essentially the same as for the normal subjects and those with congestive heart failure, differences being determined by the underlying disease state. For example, Subject 1 suffered with congestive failure due to essential hypertension and was under treatment

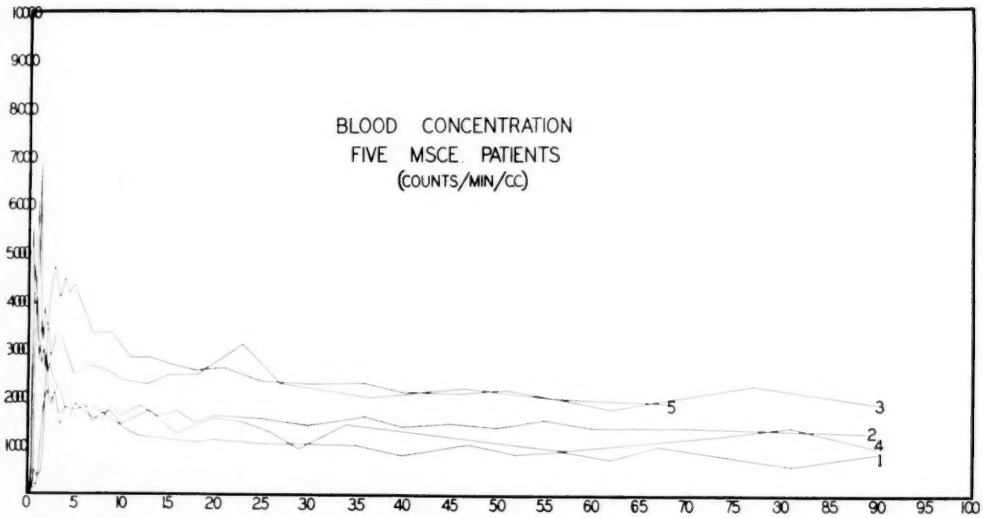

Fig. 12.—Variations in serum concentration in five patients with miscellaneous disease states (Table I).

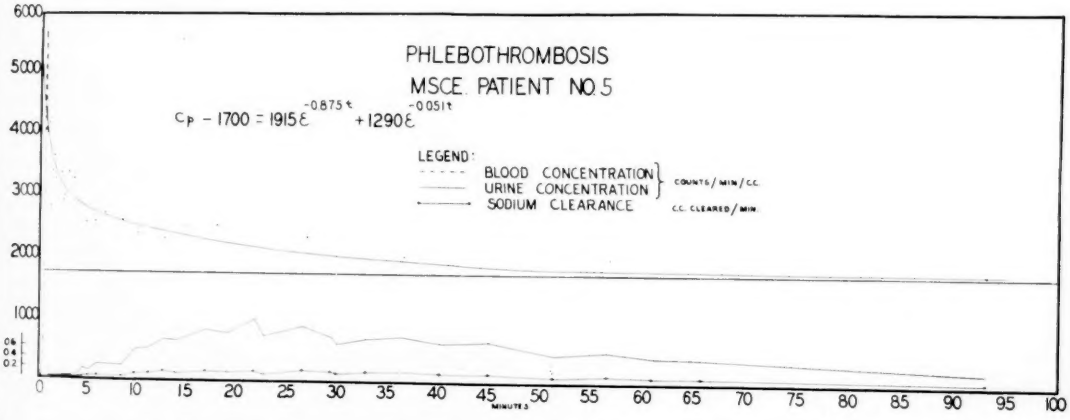

Fig. 13.—Variations in serum concentration, urine concentration, and Na^{24} clearance in a patient with chronic phlebothrombosis of both legs and moderate chronic edema. The diffusion curve for sodium and its equation are shown.

for the heart failure. She also had a fairly extensive ovarian malignancy. The variations in Na^{24} concentration in the blood and urine and in the sodium clearances were similar to those described for the group of patients with uncomplicated congestive heart failure under treatment. The patients with phlebothrombosis and sarcoidosis were not found to show any special characteristic behavior.

Subject 5 with phlebothrombosis was selected for analysis of the diffusion rates of sodium because the edema was limited to the two lower extremities and because the data were suitable for such an analysis. The curve (Fig. 13) representing variations in the serum concentration of Na^{24} is similar to that

of the normal subject and the subjects with congestive heart failure. The curve of the serum concentration may be expressed as

$$c_p - 1700 = 1915\epsilon^{-0.875\,t} + 1290\epsilon^{-0.051\,t}, \qquad (6)$$

that is, 0.875 of the plasma sodium which diffuses through area A_1 left the blood per minute, while 0.051 of the plasma sodium which diffuses through A_2 left the blood per minute.

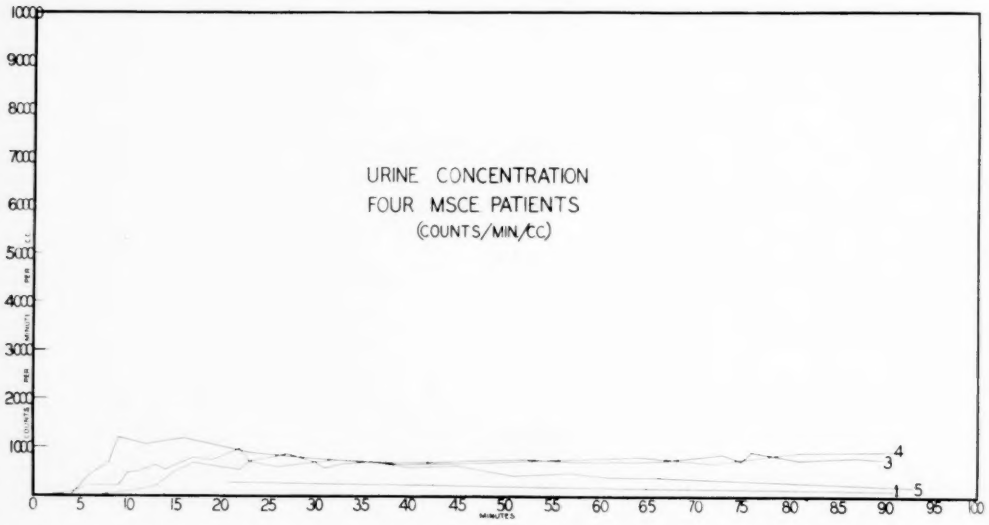

Fig. 14.—Variations in urine concentration of Na^{24} in four patients with miscellaneous disease states.

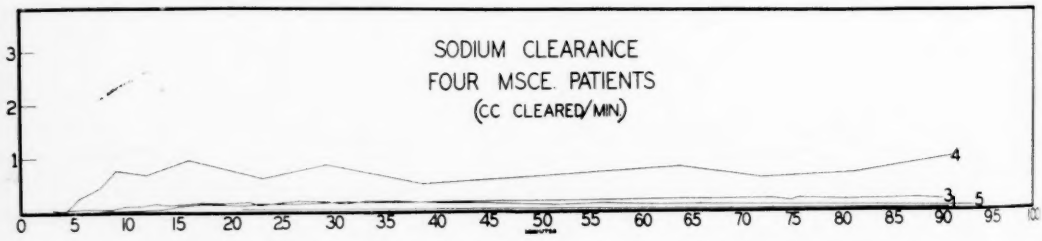

Fig. 15.—Variations in Na^{24} clearance in four patients with miscellaneous disease states. Compare these values with those of the other two groups of subjects (Figs. 4, 6, and 11).

Comment: Fever and other systemic influences from infection, malignancy, or sarcoidosis did not present any detectable influences on the diffusion of sodium through the vascular bed. The rate of excretion through the kidneys, however, might conceivably be influenced by renal damage as well as by therapy. The rates of sodium diffusion were too high to permit minor variations to manifest themselves sufficiently for detection.

The transcapillary diffusion of sodium in these subjects indicated at least two rates, a rapid and a slow one. In the subject with phlebothrombosis the rates of diffusion through areas A_1 and A_2 were of the order encountered in the two patients with congestive heart failure. The patient with phlebothrombosis

was receiving only bed rest; no therapy was directed at the edema specifically. Table II indicates the proportion of plasma and interstitial sodium diffusing through the vascular bed per minute.

Concretio Cordis and Marked Ascites: Miscellaneous Subject 7 had marked chronic cardiac tamponade with marked ascites and was receiving therapy for congestive heart failure. The therapy included the use of digitalis, mercurial diuretics, and a low sodium diet. He died at operation several days after this study and a thick fibrous sheet, which interfered markedly with diastolic filling of the heart, was found to encase the heart. He was studied in the manner previously described except that an 18-gauge needle was placed into the peritoneum so that samples of ascitic fluid could be collected at frequent intervals for study. The relative concentrations of Na^{24} in the blood and ascitic fluid are shown in Fig. 16. There was a very slow but definite rise in the Na^{24} concentration in the ascitic fluid. This rise was much slower than that observed in the urine or edema fluid of the other patients. The blood serum concentration of Na^{24} showed the usual variations.

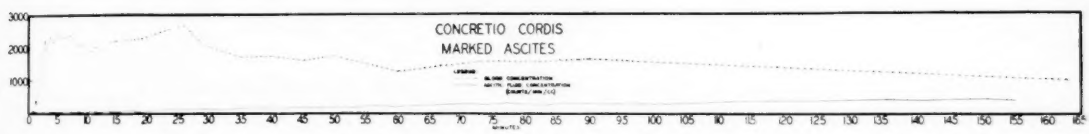

Fig. 16.—Variations in serum and ascitic fluid concentrations of Na^{24} in a patient with concretio cordis and marked ascites. Note the relatively slow increase in the Na^{24} concentration in the ascitic fluid as compared with that of the edema fluid of Subject 3 with congestive heart failure.

Comment: The relatively slow diffusion of Na^{24} into the ascitic fluid of this patient is probably due to the great mass of fluid through which the Na^{24} must diffuse, as well as a less rapid diffusion of sodium through the peritoneal membrane. The importance of the former factor is supported by the slow diffusion into the edema fluid. The tissues are richly supplied with capillaries which permit a larger area of contact with the interstitial fluid; that is, the ratio of area of capillary wall in contact with each unit volume of fluid is high. The capillary area of contact per unit volume of edema fluid is less and, therefore, a longer time is required for equilibrium to be established. In the case of the ascitic fluid the ratio of capillary wall per unit volume of fluid into which diffusion occurs is considerably less and, therefore, the slower diffusion and even longer time required for equilibrium to be established becomes evident. There is no doubt that the stirring of the edema fluid that occurs with breathing and movement of the body aids in increasing the rate with which the state of equilibrium is reached. These observations further support the evidence that several hours are required to reach equilibrium, especially in edema states, after radiosodium tracer studies are conducted.

Excretion of Na^{24} by the Sweat Glands: A normal subject (a 45-year-old white man) rested in a hot and humid air-conditioned room (temperature, 48° C.; relative humidity, 45 per cent). After the bladder was emptied he was studied in the same manner as described for the other subjects.

Results: The results are summarized in Fig. 17. The Na^{24} appeared in the sweat in seventy-five seconds after the intravenous injection. The concentration of Na^{24} was greatest in the blood and least in the urine. The concentration in the sweat tended to follow that in the blood very closely. Unfortunately the subject could not remain very long in the hot and humid atmosphere; therefore, the experiment was stopped after only thirty minutes of observation. Another sample of urine and of blood, collected about forty minutes after the subject was removed from the hot room, showed no special change in Na^{24} concentration. Concentrations of Na^{24} in the urine, blood, and sweat of normal Subject 6 are shown in Fig. 2. Six samples of sweat were measured during the period of study.

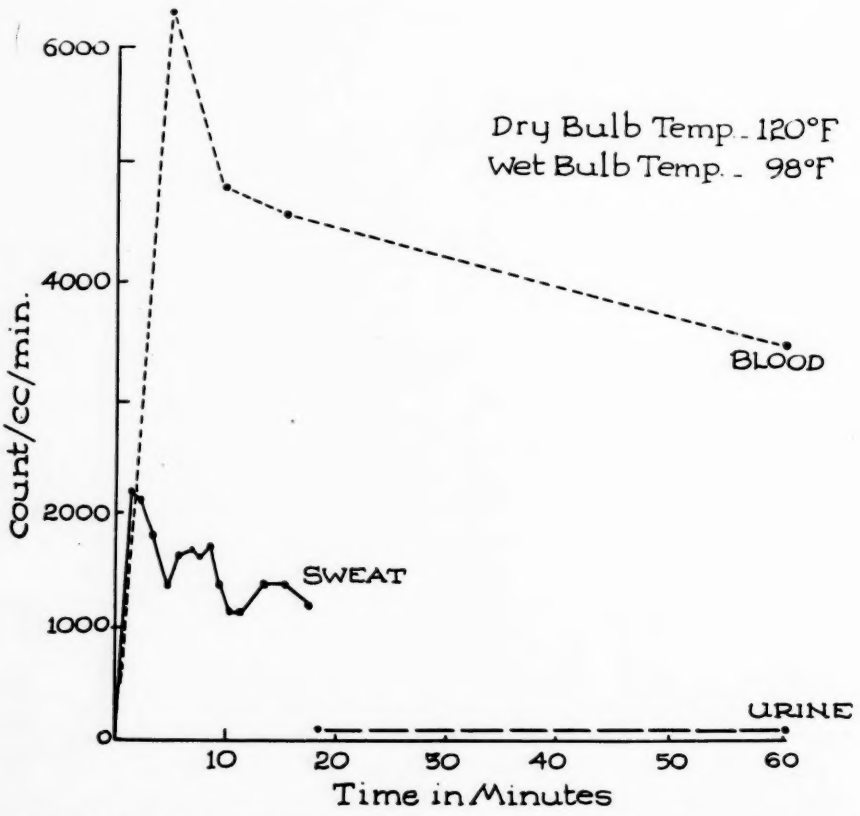

Fig. 17.—Variations in serum, sweat, and urine concentrations of Na^{24} in a normal subject who was in the process of profuse thermal sweating when the Na^{24} was injected intravenously.

Comment: These data show the rapid rate with which sodium reaches the sweat. When a correction is made for circulation time, sodium is found to diffuse from the plasma into the interstitial spaces, enter the cells of glomerulus of the sweat glands, be made into sweat, and delivered to the surface of the body in less than sixty seconds. Fig. 17 suggests many interesting concepts of integrations of urinary, sweat, and plasma sodium concentrations. Any

comments based on one experiment would have little significance. More extensive studies will be described at a later date.

GENERAL DISCUSSION

The diffusion of sodium through the vascular bed in man behaves in a manner similar to that described by Gellhorn and associates for guinea pigs and dogs.[4-6] For comparison the vascular bed can be divided into at least two areas, A_1 and A_2, through which diffusion occurs at two very different rates. The more rapid rate of sodium exchange, through A_1, takes place between the plasma and the intercellular fluid. The slower rate of exchange, through A_2, occurs between the plasma and such fluids as that of the vitreous body, aqueous humor, cerebrospinal fluid, and probably the intracellular fluid. Inspection of the regression curves shown indicates a third main, and even slower, rate of diffusion which may occur between plasma and bone, dentin, and enamel.[9] This area of the vascular bed through which this occurs might be referred to as A_3. There is no doubt that there are *many* other differences in rates of diffusion of sodium between the plasma and other structures of the body, the number being limited by the sensitivity of the method of analysis. Under different states of salt intake, nutrition, glandular secretion, vasomotor function, and disease these rates are altered. For example, there is a different rate of diffusion into edema fluid (Fig. 8) from that into ascitic fluid (Fig. 16). A generalized edema state results, therefore, in two areas in the vascular bed with different rates of diffusion. At the present time it is much better to consider the vascular bed as having two main areas with diffusion at a rapid rate and at a slow rate, these being summations of many different rates of diffusion. It is necessary to remember that this may be an oversimplification of the problem and that with further study other rates will be defined.

The rate of the circulation in man is relatively slow when compared with that which would be desirable to insure adequate mixing for proper study of a substance which diffuses as rapidly as sodium. The mixing time in the average person is so long and the diffusion of sodium out of the blood vessels so rapid that in most cases data were obtained which did not lend themselves to detailed mathematical analysis. In some experiments, however, the mixing was rapid and adequate so that a mathematical analysis was possible. Several of these were selected for such a study. The period during the first minute after the injection was not suitable for proper study of diffusion because slow mixing and rapid diffusion complicated the diffusion pattern. The dashed lines in Figs. 2, 8, 9, and 13 show the rapid increase in the concentration of Na^{24} in the blood within the first minute or two after the injection of the radiosodium, during which time the regression curves are modified to an unknown extent. The remaining portion of the curves is a more accurate representation of the facts. There is no doubt that if there are any errors, such as those due to slow mixing, they are concerned with factors which tend to make the observed b_1, the rapid rate of diffusion of sodium, smaller than it actually is. The values for b_2, the slow rates of diffusion, are most probably near the

true rate since mixing interferes little with the latter portions of the regression curves. Furthermore, since a state of equilibrium had not been fully reached by the end of ninety minutes, the equilibrium values, c_{eq} of equations (2) and (5), are not the true equilibrium values. Studies[8] with the long-life sodium, Na^{22}, in other subjects have shown the true c_{eq}, the equilibrium constant, to be near those employed in the regression curve formulas, however.

The coefficients, a_1 and a_2, of the various regression curves are dependent upon such factors as rates of diffusion, size of the areas A_1 and A_2, and the distribution of the sodium. Therefore, a_1 and a_2 can represent only roughly the degree of distribution of Na^{23} in the two compartments of the body, that is, the one which is concerned with a rapid rate of diffusion of sodium and the other with a slower rate. An index of the volume of the blood plasma should be c_o, the value of c_p at $t = 0$, but because of the difficulties introduced by slow mixing it is difficult to interpret.

The rates of diffusion appear to be quite variable among the individuals of all groups studied (Figs. 1 and 7). The rates of diffusion through areas A_1 and A_2 were definitely greater in the patients with congestive heart failure than in the normal subjects. Because of the marked variations and the relatively small number of subjects studied, it is not possible to be certain of the significance of these differences. However, as indicated previously, this is expected because of the greater absolute quantities of interstitial fluid and, therefore, of sodium in the patients with congestive heart failure. The turnover of the interstitial sodium in the patients with congestive heart failure is essentially half as rapid as in normal subjects, while the plasma sodium turnover is twice as rapid in the patients with congestive heart failure as that in the normal subjects.

The patients with congestive heart failure were too ill to permit the insertion of ureteral catheters into the pelves of the kidneys. A small urethral catheter was placed in the bladder instead. This resulted in a satisfactory study of Na^{24} excretion in the urine but did not present the advantage of studying each kidney separately. All sodium clearances for the patients with congestive heart failure represented total clearance, while those in the normal subjects represent unilateral clearance, or essentially one-half of the total clearance (Fig. 6). This should be remembered when examining the illustrations and when comparing the rates of clearance in the normal and abnormal subjects.

The radiosodium was excreted in essentially the same concentration and at the same rate by both kidneys. There was a tendency at times, however, for one kidney to excrete more rapidly than another, but this was varied throughout the period of study. Although the concentration of Na^{24} and the rate of sodium clearance tended to vary concordantly, there were sufficient variations to indicate differences in function between two contralateral normal kidneys. Although both are under the same general hormonal or chemical and nervous influences there were individual differences. Most of these differences must result from differences in intrinsic renal function. In some isolated instances these differences were considerable, although the excretion of Na^{24} usually differed very little in the two kidneys.

The rate of sodium clearance was much slower in the patients with congestive heart failure than in the normal subjects. This is particularly significant when the rapid transcapillary diffusion rates are considered. It was impossible to evaluate the differences between the two groups of subjects because the patients with congestive heart failure were receiving treatment for the failure. This treatment included dietary restriction of sodium, a procedure normally associated with a decrease in renal excretion of sodium. Nevertheless, these differences are largely due to the congestive heart failure, as evidenced by previous studies[7] with Na^{22} and to the fact that three of the patients with a low sodium clearance had not been on sodium restriction for more than twenty-four hours before the study was begun. The long life of Na^{22} made it possible to control sodium intake, therapy, diet, and other factors in order to evaluate better the disturbances in sodium excretion in congestive heart failure. Unfortunately, this was not possible in these studies with Na^{24}.

Table I shows estimations of sodium space. A sodium space calculated within one or two hours after injection cannot be accurate. This is obvious from the preceding discussion of the rapid rate of diffusion of sodium and the relatively long time required for a state of equilibrium or "complete mixing" of the Na^{24} with all of the Na^{23} distributed through the body to occur. The inaccuracy of such a calculation is further indicated by Table I. The sodium space calculated from data at the sixtieth minute after injection of the Na^{24} was lower than that calculated at the ninetieth minute. Furthermore, radiosodium cannot be employed to measure extracellular water volume with any degree of accuracy because a fairly large portion of the body sodium is not in the extracellular water. The method becomes even more inaccurate in disease states, especially in those associated with edema. The values of sodium space presented in Table I are intended only to illustrate the possible error of such a calculation and not to present absolute values of sodium space. The lower equilibrium level, c_{eq}, for the patients with congestive heart failure and the larger values obtained for the estimated sodium space in these patients are quantitative evidence of the large edema fluid volume accompanying congestive heart failure.

The rapid rate and high concentrations with which Na^{24} appeared in sweat is further evidence in support of the rapid rate of diffusion of sodium across the vascular wall. The relationships of sodium in the sweat, urine, blood, and edema fluid are under study. The single study presented is not sufficient to warrant any definite physiologic conclusions, although many ideas are suggested.

Finally, it is important to note that the rapid rates of sodium and water diffusion and the tremendous quantities concerned make the problem of the mechanism of edema assume a different pattern. Sufficient evidence has not been gathered yet to make possible a proper appreciation of the role of the rapid and large turnover of sodium and water in edema formation. It is obvious that slight disturbances in sodium diffusion or excretion might result in a rapid and marked accumulation of edema fluid. These factors are now under observation.

SUMMARY

Normal subjects, patients with congestive heart failure under treatment, and patients with other disease states were studied following a single intravenous injection of Na^{24} to learn the rate of diffusion of sodium through the vascular wall and its rate of excretion into the urine and sweat.

Sodium diffuses through the vascular wall with at least two rates, *rapid* and *slow*. These rates of diffusion of sodium in man are similar in quantity and quality to those described by Gellhorn and associates for guinea pigs and dogs. In a normal subject the average rate of diffusion indicated that about 32 per cent of the total plasma sodium diffused out of the blood every minute. This approaches a diffusion across the vascular wall equal to about 18 pounds of sodium or 50 pounds of sodium chloride per day.

The sodium that diffuses at a rapid rate is mainly concerned with an exchange between the plasma and interstitial fluid, while that which diffuses at the slow rate is probably concerned with exchanges between the plasma and such fluids as the vitreous body, aqueous humor, and cerebrospinal fluid. The regression curves observed suggest a possible third or slower rate of sodium diffusion such as might be expected for bone, dentin, and enamel already saturated with sodium.

The individual subjects in all groups showed a considerable spread in the blood and urine concentrations and rates of sodium cleared by the kidneys. The patients with congestive heart failure with treatment, including dietary restriction of sodium, showed a more rapid rate of sodium diffusion from the vascular bed than did the normal subjects. This was also true for a patient with phlebothrombosis receiving only bed rest therapeutically. The rate of turnover of the interstitial fluid sodium was 50 per cent as rapid in the patients with congestive heart failure as in the normal subject, while the plasma sodium of the patients with congestive heart failure turned over twice as rapidly as that of the normal subject.

The rapid rates of diffusion and the large quantities of sodium involved make it necessary to reconsider the role of sodium in edema formation. Relatively slight changes in the rates of sodium diffusion from the blood with a disturbance in its excretion in the urine might result in rapid and marked edema formation.

The rate of sodium clearance in the patients with congestive heart failure was considerably less than that in the normal subjects. These differences were not due entirely to a dietary restriction of sodium intake.

Simultaneous study of each kidney showed a tendency for the renal excretion of sodium to be similar bilaterally. There was sufficient qualitative and quantitative difference, however, to indicate intrinsic renal differences in the excretion of sodium. In some instances these differences were considerable.

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MANNITOL: KINETICS OF DISTRIBUTION, EXCRETION, AND UTILIZATION IN HUMAN BEINGS

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MANNITOL is in current clinical use as a measure of the rate of glomerular filtration[14] and of the volume of extracellular fluid.[11] It is also recommended as a harmless osmotic diuretic.

The availability of a method[4] for the determination of plasma mannitol, which, especially at low concentrations, is more precise than procedures formerly in use, enabled a study of the distribution, excretion, and utilization of mannitol after intravenous injection in human beings. This study is based on concepts previously developed[5-8] and applied to creatinine, xylose, and galactose. Since these concepts have been presented in detail and verified experimentally, we limit our prefatory discussion to general terms and to specific symbolisms used in this report.

If the rate of urinary excretion, y, of a substance (milligrams per minute) is proportional to the concentration, x, of this substance in plasma (milligrams per cubic centimeter), the excretion constant A can be calculated. This constant A is usually interpreted as the volume of plasma cleared of the substance per unit time and is called clearance (cubic centimeter per minute). It may be interpreted also as the rate of excretion per unit plasma concentration, or, in short, the specific rate of excretion. When the substance in question is disposed of wholly by renal excretion and when the rate of diffusion of this substance into body fluid is such that the amount lost before equilibration is negligible, the constant A can be calculated from serial determinations of the concentration of the substance in plasma. The calculation can be made graphically from a plot of plasma concentration x on semilogarithmic paper. This plot will follow a straight line with slope α. The initial concentration a is obtained directly by extrapolating the line to the time of injection. This value a corresponds to a theoretic concentration of the substance in the plasma under the assumption of mixing and equilibration at the time of injection. The dose, G, being known, the volume of distribution, V, of the substance is determined from G and a. The excretion constant A is obtained from G, a, and slope α. The formulas are collected for convenience in Table I, while Fig. 1 illustrates the procedure.

When the rate of equilibration is such that significant disposal of the substance takes place before equilibrium is established, the values V and A must be regarded only as first approximations, identified by a subscript 1, as A_1 and V_1. If the pre-equilibrium plasma concentrations are known, as in the experiment shown in Fig. 1, the slope β and the extrapolated concentration b can be determined from the excess of plasma concentration above the line of the slope α.

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With these new constants a second approximation, A_2, and a new V, V_2, are then calculated (Table I). The value A_2 measures the total specific rate of disposal of the substance by urinary and other possible pathways and represents total plasma clearance.

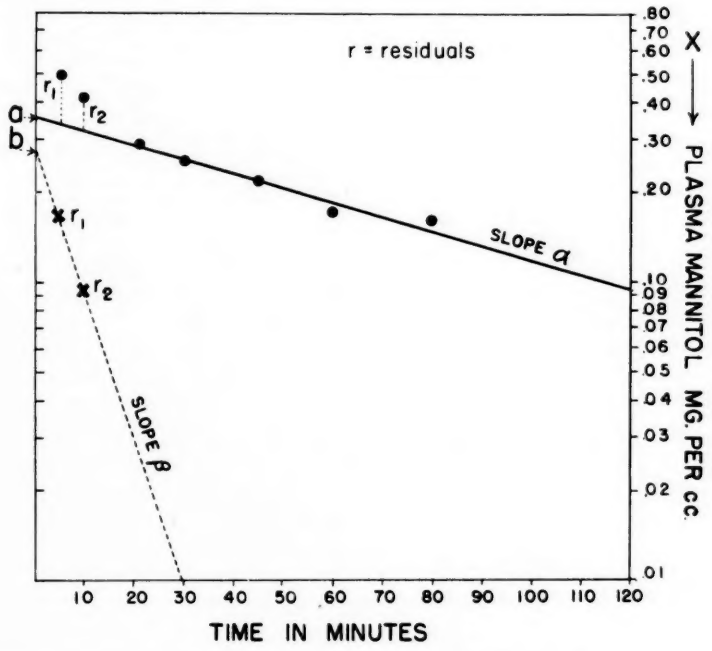

Fig. 1.—Graphic solution of the equation of the plasma disappearance curve, $X = ae^{-\alpha t} + be^{-\beta t}$ (No. 77).

$a = .355$

$$\alpha = \frac{\ln\ 0.355 - \ln\ 0.10}{113.5} = \frac{1.2669}{113.5} = 0.01116$$

$b = .285$

$$\beta = \frac{\ln\ 0.285 - \ln\ 0.01}{29.6} = \frac{3.3499}{29.6} = 0.1131$$

The extraurinary pathways of disposal lie either in metabolism or extrarenal excretion. The sum of these processes is referred to herein and in earlier reports as utilization. If the substance (1) is both excreted in the urine and utilized and (2) disappears exponentially from plasma, the former value A_1 must be regarded as the sum of two constants, A_3 and B_3, of which A_3 is another approximation to the renal plasma clearance and B_3 is the utilization constant. This constant represents the rate of utilization per unit plasma concentration, or, in brief, the specific rate of utilization.

The value of A_3 is calculated from the amount, E, of the substance which appears in the urine. When the time of urine collection is prolonged and renal function intact, so that excretion is virtually complete in the time of collection, A_1 is calculated as shown in Table I. When the period of collection is short, so that excretion is incomplete, an exponential term is introduced leading to the value A_3' (Table I).

TABLE I. FORMULAS USED IN THIS REPORT

(1) $y = Ax$

(2) $\int y\,dt = A \int x\,dt$

(3) $x = ae^{-\alpha t} + be^{-\beta t}$

(4) $A_1 = \dfrac{\int y\,dt}{\int x\,dt} = \dfrac{G}{\int ae^{-\alpha t}\,dt} = \dfrac{G}{\left(\dfrac{a}{\alpha}\right)}$

(5) $A_2 = \dfrac{G}{\int (ae^{-\alpha t} + be^{-\beta t})\,dt} = \dfrac{G}{\dfrac{a}{\alpha} + \dfrac{b}{\beta}}$

(6) $A_3 = \dfrac{E}{\dfrac{a}{\alpha}}$

(7) $A_4 = \dfrac{E}{\dfrac{a}{\alpha} + \dfrac{b}{\beta}}$

(8) $A_3' = \dfrac{E_t}{\dfrac{a}{\alpha}(1 - e^{-\alpha t})}$

(9) $A_4' = \dfrac{E_t}{\dfrac{a}{\alpha}(1 - e^{-\alpha t}) + \dfrac{b}{\beta}(1 - e^{-\beta t})}$

(10) $\dfrac{A_3}{A_1} = \dfrac{A_4}{A_2} = \dfrac{E}{G}$

(11) $B_4 = A_2 - A_4$

(12) $V_1 = \dfrac{A_1}{\alpha}$

(13) $V_2 = \dfrac{A_2(\beta^2 a + \alpha^2 b)}{(\beta a + \alpha b)^2}$

(14) $\dfrac{A_1}{A_2} = 1 + \dfrac{\alpha}{\beta} \cdot \dfrac{b}{a}$

(15) $\dfrac{V_1}{V_2} = 1 + 2\,\dfrac{\alpha}{\beta} \cdot \dfrac{b}{a}$ approximately

The formulas used herein and in earlier reports are summarized in this table for convenience. The meanings of the symbols are explained in the text.
G, Quantity injected in milligrams.
E, Total urinary excretion, milligrams.
E_t, Quantity excreted in urine in time, t.

In the calculations of A_3 and A_3', the diffusion of the substance is neglected. If both diffusion and extraurinary disposal are taken into consideration, a value, A_4 (and also A_4' for a short period of collection), can be calculated as shown in Table I. The value A_4 is equal to renal plasma clearance of a substance which is excreted in urine and which is also utilized and subject to pre-equilibration disposal.

Successive calculations of these different values of A give explicitly the errors introduced in the estimate of renal clearance. The ratio $\dfrac{A_1}{A_2}$ gives the relative error introduced in the estimate A_1 by neglecting diffusion. The ratios $\dfrac{A_1}{A_3} = \dfrac{A_2}{A_4}$ give the relative error introduced in the estimates A_1 and A_2 by assuming no utilization. The ratio $\dfrac{A_1}{A_4}$ gives the relative error introduced in the estimate A_1 by neglecting diffusion and by assuming no utilization.

The utilization constant B already defined is obtained from the difference $(A_2 - A_4)$ (Table I).

PROCEDURE

Seventy experiments were performed on fifty-six subjects, thirty-nine of whom suffered from some form of renal disease. Twenty cubic centimeters of a

25.5 per cent solution of mannitol* were rapidly injected intravenously. Plasma samples were obtained after equilibration in some experiments and both before and after equilibration in other experiments. The number of plasma samples varied from three to seven. Urine was collected for short periods (averaging 105 minutes) and for long periods (nine to twelve hours). The distribution of procedures is shown in Table II.

TABLE II

PLASMA SAMPLING	URINE COLLECTION			TOTALS
	NONE	SHORT	LONG	
After equilibration	36	13	7	56
Before and after equilibration	2	7	5	14
Total	38	20	12	70

Data from one of the five experiments with long urine collections in which plasma was obtained before and after equilibrations are shown in Fig. 1, from which the course of other experiments can be visualized. In each of ten subjects the experiments were repeated once, and in each of two other subjects the experiments were done three times. Two of the repeated experiments cannot be averaged because of changes in the subject's renal excretory function.

Plasma mannitol clearance, C, was determined in patients independently of this procedure on thirty occasions (Table IV). These determinations were done by the conventional method of intravenous infusion, plasma sampling, and urine collection by catheterization and by bladder washing. A value termed C' was calculated from the curve of plasma concentrations and concurrent excretion of mannitol in voided urine in five experiments. This value is equivalent to conventional clearance determination, but, in contrast with C in this series, the determination C' is simultaneous with the experiment rather than an independent observation.

RESULTS

Volume of Distribution.—Mean V_I in the whole group is 14.2 liters, while mean body weight, W, is 66.7 kilograms, mean surface area, S, is 1.74 square meters, and mean developmental level, $D.L.$,[16] is 166. The correlation coefficient between V_I and W is 0.49; that between V_I and S, 0.53; and between V_I and $D.L.$, 0.53. The relation between V_I and $D.L.$ is shown in Fig. 2, which demonstrates that, on an average, V_I is greater in patients than in normal subjects at equal developmental levels. The differences in the means of V_I between patients (14.9 liters) and normal subjects (12.6 liters) is 2.3 liters, which is found to be 2.8 times the standard error of the difference and is, therefore, significant. The importance of the difference is still more apparent when it is related to body weight or developmental level in the two groups, mean W being 65.9 kilograms in patients and 68.8 kilograms in the normal group and mean $D.L.$ being, respectively, 164 and 170.

*We acknowledge the generosity and interest of Dr. Carl Beyer and Dr. William Boger, of Sharp & Dohme, Inc., Philadelphia, Pa., and the skillful assistance of Dr. L. Underwood and Miss E. Davy, R.N.

The second approximation of volume of distribution, V_2, is calculable in those cases in which plasma mannitol was determined before equilibration. In this group V_1 averages 13.8 liters and V_2, 12.3 liters (Table III). The difference is 1.5 liters and the ratio V_1:V_2 is 1.12. The excess of the ratio V_1:V_2 over unity measures the error in the estimate of volume of distribution occasioned by

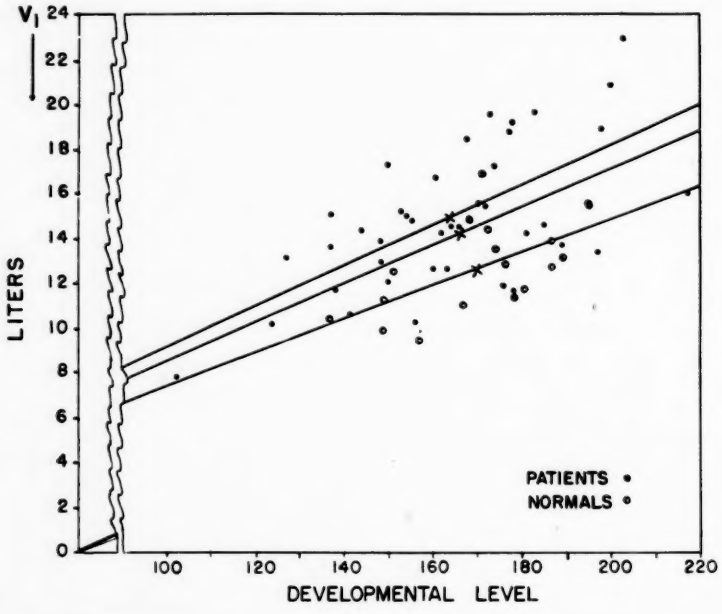

Fig. 2.—Relation between V_1 and developmental level, *D.L.*, in patients and in normal subjects. The means for each group are indicated as *X*. These points are joined to zero by straight lines. The upper line indicates the relation of V_1:*D.L.* in patients; the lower line, the relation in normal subjects; and the middle line, that in the whole group.

TABLE III. CONSTANTS OF THE EQUATION OF PLASMA MANNITOL DISAPPEARANCE

$x = ae^{-\alpha t} + be^{-\beta t}$ AND DERIVED VALUES OF P, V_1 AND V_2

NO.	a	α	b	β	P	V_2	V_1
60	35.5	0.0049	40.0	0.290	6.76	13.8	14.4
61	36.8	0.0043	15.0	0.082	9.85	13.3	13.9
62	30.4	0.0056	25.0	0.153	9.21	15.8	16.8
63	43.1	0.0037	40.0	0.150	6.14	11.3	11.8
64	49.5	0.0075	35.0	0.178	6.03	9.7	10.3
65	37.6	0.0092	34.5	0.096	7.1	11.7	13.6
66	36.0	0.0095	19.2	0.100	9.2	12.9	14.2
68	35.2	0.0092	59.0	0.076	5.4	10.2	14.5
69	22.3	0.0097	14.0	0.091	14.0	20.3	22.9
77	35.5	0.0112	28.5	0.113	8.0	12.4	14.4
78	40.5	0.0117	44.5	0.119	6.0	10.4	12.6
79	38.2	0.0121	23.0	0.098	8.3	11.7	13.4
80	52.0	0.0131	36.5	0.128	5.8	8.6	9.8
81	45.6	0.0112	42.5	0.155	5.8	10.5	11.2
Means					7.68	12.33	13.8

Constants obtained in experiments in which plasma mannitol concentrations were known before and after equilibration. The value *P* (Dominguez) is the volume into which injected mannitol diffuses so rapidly that is is identified in earlier reports as equivalent plasma volume. In these experiments the volume *P* averages about 2.7 times true plasma volume as estimated from body weight and 0.62 times the final volume of distribution, V_2. The significance of the other constants is explained in the text.

neglecting diffusion prior to equilibration (Table I). V_2 is, therefore, the more exact measure of volume of distribution. If this value of $\frac{V_1}{V_2}$ holds for the whole group, V_2 averages 13.3 liters (20 per cent of body weight) in patients and 11.3 liters (16 per cent of the body weight) in normal subjects.

Equilibration of Mannitol in Body Fluid.—Since mannitol is subject to continuous disposal, the equilibration of mannitol in body fluids is dynamic. After a single injection, the first gradient is from plasma to interstitial fluid, and the second, from body fluid to plasma, from which mannitol is excreted in urine. The time of equilibration marks completion of the first phase, after which disappearance of mannitol from plasma becomes simply exponential. Plasma samples were obtained at or about twenty minutes after injection in thirty-nine subjects. The weighed mean time of equilibration is twenty-seven minutes, equilibrium being established at twenty minutes in nineteen experiments, at twenty-five in one, at thirty in ten, and at forty in nine. In four experiments in which the first blood sample was taken at thirty minutes, equilibration had already been established. The mean time of equilibration in the fifteen experiments in which blood samples were obtained shortly after injection (as in Fig. 1) was twenty-eight minutes, the times being twenty minutes in six, twenty-five minutes in one, thirty minutes in three, thirty-five minutes in four, and forty-five minutes in one.

Thus, mannitol was equilibrated in body fluids at a mean interval of twenty-seven to twenty-eight minutes after injection, but it is well to bear in mind that in ten of fifty-eight experiments the time of equilibration was forty minutes or more.

Renal Excretion.—The first approximation of the specific rate of mannitol disposal A_1 is compared with the independently determined renal plasma clearance, C, in Table IV. The ratio of the mean of A_1 and C in this group is 1.44. This estimate of the ratio A_1:C may be somewhat affected by changes in function between measurements of A_1 and C. But the inequality of the means indicates that a considerable portion of injected mannitol is disposed of by avenues not taken into account in assuming the equivalence of A_1 and C in the estimation of A_1. There must be loss before equilibration, or utilization before and after equilibration, or a combination of these factors.

That disposal before equilibration should not be as great as the excess over unity of A_1:C indicates is suggested by the rapidity of mannitol diffusion. The effect of diffusion from plasma on the estimate of volume of distribution is noted in the foregoing in comparing V_1 and V_2. Similarly, the effect of this diffusion on the estimate of specific disposal rate is observed in the ratio of mean A_1:A_2 (Table V) which is 1.08, an excess over unity of the same order as that of the ratio V_1:V_2. Loss of injected mannitol before equilibration is, therefore, not negligible.

However, if such loss were the only factor determining the excess of A_1 over C', the ratio A_2 to C' would be unity. Actually, the ratio of the means is 1.23 (Table V), from which it follows that the specific rate of disposal of injected mannitol involves also a considerable utilization of this substance.

TABLE IV. COMPARISON OF THE MANNITOL CLEARANCE C TO THE FIRST APPROXIMATION A_1 TO THIS CLEARANCE

C SMALLER THAN 70			C GREATER THAN 70		
NO.	A_1	C	NO.	A_1	C
2	95	30	3	182	124
4	92	67	5	157	84
7	56	40	14	145	80
8	64	68	22	109	88
9	60	60	25	132	85
11	67	48	28	103	78
19	58	35	35	153	140
20	82	54	44	139	88
27	103	60	46	163	125
29	84	64	51	188	124
33	72	30	65	125	83
34	55	23	66	135	97
38	23	21			
43	27	17			
60	71	66			
62	94	50			
63	44	28			
64	77	50			
Mean	67.9	46.2		144.3	99.7

Mean $A_1 = 1.44 \times$ mean C in patients whose level of C is less than 70, and 1.45 when C is greater than 70. The mean of the ratios A_1: C for patients is 1.62 when C is less than 70 (*S.D.*, 0.55) and 1.47 (*S.D.*, 0.23) when C is greater than 70. The difference between these means is not significant (difference/standard error of difference is less than 1). The greater variability of data in patients in whom C is less than 70 is attributed in part to changes in function which may have occurred in the interval between measurements of A_1 and C. Such changes are less marked when function is initially close to normal with C greater than 70.

TABLE V. SUCCESSIVE APPROXIMATIONS TO THE CLEARANCE OF MANNITOL COMPUTED FROM THE PLASMA CURVE ALONE (A_1 AND A_2), FROM THE PLASMA CURVE AND SHORT INTERVAL OF URINE COLLECTION (A_3' AND A_4') AND FROM THE PLASMA CURVE AND LONG INTERVAL OF URINE COLLECTION (A_3 AND A_4). COMPARISON WITH INDEPENDENT CLEARANCE C AND SIMULTANEOUS CLEARANCE C′

NO.	A_1	A_2	A_3'	A_3	A_4'	A_4	C′	C
60	71	69	66	–	62	–	–	66
62	94	91	50	–	47	–	–	50
63	44	43	44	–	40	–	–	28
64	77	75	48	–	45	–	–	50
65	125	115	56	–	48	–	–	83
66	135	128	78	–	71	–	–	97
77	161	149		137		126	125	
78	147	133		107		96	100	
79	162	150		137		128	111	
80	128	120		111		104	99	
81	125	117		116		108	112	
Mean	145	134		122		112	109	

Data from patients (Nos. 60 to 66) and normal subjects (Nos. 77 to 81). Data from patients are not averaged because of their variability. The means of the ratios of A in normal subjects are as follows: A_1:A_2, 1.29; A_2:A_4, 1.20; A_3:A_4, 1.08; A_4:C', 1.03; A_1:A_2, 1.08; A_1:A_3, 1.19; A_1:C, 1.33. The specific rate of utilization B_4' in experiments with short periods of urine collection averages 34 and is widely variable. In normal subjects, with prolonged urine collection, mean $B_4 = 21$.

When this utilization is taken into account, as it is in calculating A_3 and A_3', neglecting pre-equilibrium disposal, the means of these values only slightly exceed the means of C and C' (Table V) (ratio of mean A_3:C' is 1.12). This excess over unity measures the degree to which the estimate of renal excretion of mannitol (A_3 or A_3') is affected by pre-equilibration disposal. When this loss is also accounted for, as it is in calculating A_4 and A_4', the correspondence

of A_4 and A_4' with C and C' is seen to be close (Table V) (ratio of mean A_4:C' is 1.03). The discordance of three ratios A_4:C (Nos. 63, 65, and 66) can be attributed to changes in renal function which may have occurred in the interval between the determination of C and that of A. This supposition is supported by the fact that the concordance with the simultaneous observations C' is nearly exact.

Thus, specific rate of renal mannitol excretion obtained from serial determinations of plasma mannitol as A_4 or A_4' corresponds with values for renal plasma mannitol clearance, measured as C or C'. Both utilization and diffusion must be taken into account in the estimation of renal mannitol clearance from serial analyses of plasma after injection of mannitol.

Utilization.—The difference $(A_2$-$A_4)$ establishes the existence of mannitol utilization and measures the specific rate of this utilization, B. Direct evidence of this extraurinary disposal is obtained (Table VI) from observation of urinary excretion of mannitol over a long interval of time in subjects with normal or nearly normal renal functions. The mean recovery of injected mannitol is 79 per cent. Recoveries of 81 and 89 per cent were obtained by Smith, Finklestein, and Smith[14] in ten and one-half hours of urine collection, and recoveries of 80, 85, and 90 per cent in twenty-four hours by Berger, Farber, and Earle.[2]

TABLE VI. RECOVERY OF MANNITOL IN THE URINE; 5,100 MG. INJECTED

SUBJECT	TIME (MIN.)	AMOUNT OF MANNITOL (MG.)	PER CENT RECOVERED
70	720	3,595	70.5
71	720	4,329	84.9
72	660	4,108	80.5
73	660	3,172	62.2
74	660	3,843	75.4
75	720	3,852	75.5
76	720	3,986	78.2
77	540	4,331	84.9
78	720	3,690	72.4
79	785	4,350	85.3
80	540	4,411	86.5
81	720	4,702	92.2
Mean	680.4		79.04

The validity of a nine- to twelve-hour urine collection as a measure of total urinary excretion can be shown by the following example (No. 77). The value of a is found as 0.355 mg. per cubic centimeter; α, .0112; b, .285; β, 113; t, 540 minutes; αt, 6.048; βt, 61.02; and $1\text{-}e^{-6.048}$, .99764. Amount excreted in 540 minutes is 4,331 milligrams. Assuming the 540 minute excretion to be total excretion, E, 4,331 = A_4 (34.22) and A_4 = 126.6 cubic centimeters per minute. Taking now 4,331 only as the amount excreted, E_t, in 540 minutes, and calculating A_4', as is done when urinary excretion is incomplete, we have 4,331 = A_4' (31.70 × .99764 + 2.52) from which $A_4' = \frac{4331}{34.15} = 126.8$ cubic centimeters per minute. The correspondence of A_4 and A_4' shows that excretion in 540 minutes is a close measure of total excretion. Actually, with A_4' = 126.8, the total cal-

culated excretion will be $126.8 \times 34.22 = 4{,}339$ milligrams. The difference between this and the amount excreted in 540 minutes is only 8 mg., or 0.2 per cent of E.

Indeed, when αt is greater than 4.667, the factors $1\text{-}e^{-\alpha t}$ and $1\text{-}e^{-\beta t}$ can be neglected, being less than 1 per cent of unity. As a matter of fact, the smallest value of αt in the experiments of Table VI is 5.83. Consequently, the amounts excreted in the experiments of Table VI are less than 1 per cent smaller than the total excretion, and the mean recovery of 79 per cent can be considered equal to total recovery of injected mannitol.

The specific rate of mannitol utilization is measured as B. In experiments of short duration, this constant is estimated as 34, mean of B_4', and in experiments with long urine collection, as 21, mean of B_4 (Table V). The greater variability of data in the shorter experiments leads us to believe that the estimate of B from the longer experiments (B_4) is the more representative of the specific rate of mannitol utilization in human beings.*

DISCUSSION

The present investigation was aimed at evaluating renal function by the disappearance of intravenously injected mannitol from plasma without collection of urine in the manner suggested by Barnett[1] for inulin and by Newman, Bordley, and Winternitz[11] for mannitol.

In proposing this procedure as a measure of glomerular filtration, the latter authors note that it is based on the assumptions, "(1) that mannitol is dissolved in a space approximating the extracellular fluid of the body; (2) that an equilibrium of concentration is established throughout the fluid; and (3) that loss of mannitol is only through excretion by the kidneys." The first of these assumptions is supported by our data, since the volume V_2 corresponds well with other estimates of extracellular fluid volume. The third assumption is negated by the demonstrated utilization of mannitol. The view implicit in the second assumption that excretion of mannitol before equilibration is negligible is also controverted, since neglect of this disposal introduces errors of about 10 per cent in the estimates of V and A. It is, therefore, impossible to arrive at an accurate estimate of renal plasma mannitol clearance when only the dose injected, G, and the slope, α, are known. It has recently been shown[2, 4, 10, 13] that the plasma clearance of mannitol is about 90 per cent of that of substances presumably excreted by glomerular filtration without tubular reabsorption.

However, with the additional safeguards[6] of a knowledge of pre-equilibrium plasma concentrations and of the amount excreted, renal plasma mannitol clearance can be calculated as A_4 or A_4'. Unfortunately for convenience the procedure then requires at least four and preferably five plasma samples, and, because of the individual variability of the specific utilization constant B, a prolonged collection of urine.

*The ratio of the clearance determined by the infusion method of Earle and Berliner[6] to conventional plasma mannitol clearance is found by Berger, Farber, and Earle[2] to be 1.12. If renal plasma mannitol clearance of these subjects is taken as 110, the specific rate of utilization is $(110 \times 1.12 - 110) = 13$.

The behavior of intravenously injected mannitol in human beings resembles that of xylose in dogs.[6] Like xylose, mannitol is distributed in a comparable volume of body fluid, is largely excreted in the urine by glomerular filtration and slight reabsorption,[12] and is also subject to utilization. On the basis of body weight, the utilization constant B of mannitol in normal human beings is 0.30, while that of xylose in dogs is 0.97. This difference in the constants emphasizes the value of the calculation of B in estimating utilization. The mean per cent recovery of xylose in dogs averages 60 per cent and that of mannitol in human beings, 80 per cent, from which it appears that the two substances are utilized in comparable proportion. However, the specific rate of utilization of xylose per unit body weight is three times that of mannitol.

It is interesting to note that in dogs the specific rate of utilization of sorbitol, the isomer of mannitol, is even greater than that of xylose. If we assume a body weight of 15 kilograms and an A of 35 in the experiments of Todd, Myers, and West,[15] the estimate[5] of B for sorbitol is 3.5 per kilogram of body weight.

The pathway of utilization of mannitol is unknown. It seems unlikely that mannitol should be subject to significant extrarenal excretion; therefore, utilization of mannitol, like that of xylose, may be taken as due to metabolic conversion.

Most published studies on the metabolism of mannitol are based on feeding experiments. These studies have shown that, in the rat, ingestion of mannitol leads to storage of liver glycogen.[3, 15] In the rabbit, mannitol does not relieve the symptoms of insulin shock.[3] Since mannitol is readily subject to bacterial degradation, it has been assumed that most of the metabolic utilization of ingested mannitol is due to absorption of products of bacterial action. However, the comparatively rapid utilization of mannitol we describe suggests that the results of feeding experiments reflect parenteral conversion rather than enteral breakdown.

SUMMARY AND CONCLUSIONS

The disposal of a single intravenous injection of mannitol in human beings is described on the basis of concepts formerly applied to creatinine, xylose, and galactose. The method of calculation is presented.

The volume of distribution of mannitol in body fluids is estimated as 16 per cent of body weight in normal subjects and 20 per cent in patients suffering from cardiac and renal disease. When no allowance is made for disposal of mannitol before equilibration in body fluids, the estimates of distribution volume are increased by 12 per cent. The time of equilibration of mannitol in body fluids averages 27.5 minutes, but in ten of fifty-eight experiments this interval was prolonged to forty minutes or more.

Utilization of mannitol, presumably metabolic, is shown to proceed at a rate which, like the specific rate of excretion, can be expressed as a specific rate of utilization in milligrams per minute per unit plasma concentration. This specific rate is estimated as about 21 in normal human beings. In subjects with normal renal function, excretion of injected mannitol is completed

in nine to twelve hours. The proportion excreted in this time averages 79 per cent of the amount injected.

The estimate of renal plasma mannitol clearance from the exponential plasma disappearance curve and the dose alone after a single intravenous injection is inadequate. In normal subjects the error is found to be about 30 per cent when no allowance is made for pre-equilibration disposal and for utilization. The error is reduced to 20 per cent by taking into account pre-equilibrium disposal, and to about 10 per cent when allowance is made for utilization. Although variability of data is considerable in patients suffering from diseases which alter renal function, the mean error of the estimate of mannitol clearance from the plasma disappearance curve is negligible when allowances are made for both utilization and pre-equilibration disposal. In practice, this estimate requires analysis of serially obtained plasma samples and of urine collected over a long interval.

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STUDIES IN SERUM PROTEINS

I. The Chemical Estimation of Albumin and of the Globulin Fractions in Serum

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THE increasing clinical significance being attached to the various components of the serum proteins as revealed by electrophoresis necessitates a simple, rapid procedure for estimating these fractions. The characteristics of the Tiselius electrophoresis apparatus make it impractical for most clinical laboratories. As a result, fractionation studies cannot be performed in many cases where they would be desirable.

A simple chemical fractionation method which approximated the results obtained by electrophoresis is reported. This chemical procedure requires no special equipment and can be performed in several hours of actual working time using serum samples of nominal size.

MATERIALS AND METHODS

Four human sera, one normal and three abnormal, were fractionated by both electrophoretic and chemical methods. Five samples of animal plasma were also studied; these were obtained as desiccated powders and were reconstituted with distilled water. In the case of the animal plasma samples it was necessary to undertake a preliminary removal of fibrinogen; this was done by the addition of purified clotting thrombin. Three of the animal samples were of sheep plasma, and one each of equine and porcine plasma.

Analyses‡ on the human sera and on the animal plasma samples were carried out electrophoretically; in calculating the percentage composition of the fractions of these samples (Table II) a suitable correction has been made for fibrinogen.

Chemical procedures utilized for determining the various protein fractions were as follows:

1. Total protein was determined by the biuret reaction as modified by Weichselbaum.[1]

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‡Analyses on human sera carried out by Dr. Donald Buchanan; those on animal plasma samples, by Dr. J. D. Perrings.

We are greatly indebted to Dr. E. S. G. Barron and Dr. Donald Buchanan, of the Department of Medicine, University of Chicago, for their kind cooperation in making it possible to check our human sera analyses with electrophoretic runs, and to Dr. R. L. Kutz and Dr. J. D. Perrings of the Biochemical Section, Armour Laboratories, Armour and Company, who provided us with liberal samples of desiccated animal plasma of precisely known electrophoretic composition.

2. Albumin plus alpha globulin is equivalent to the "albumin" fraction obtained by employing Kingsley's modification of Howe's method to precipitate "globulin."[2] The estimation was finally performed by the biuret method.
3. Albumin values were obtained by the methanol fractionation procedure of Pillemer and Hutchinson.[3] The methanol filtrate was analyzed for protein by the biuret reaction.
4. Gamma globulin was separated from serum by a modification of the method of Cohn and co-workers.[4] A solution of the precipitating reagent was prepared by adding 2.5 ml. of 1 N. sulfuric acid to 100 ml. of 2.78 M. ammonium sulfate. Five-tenths milliliter of serum was placed in a graduated 10 ml. Kolmer centrifuge tube. Four milliliters of precipitating solution were then placed in a doubly tied cellophane bag suspended in the tube and centrifuged until 0.5 ml. of ammonium sulfate solution had been added to the serum. The rate of centrifugation was adjusted so that about two hours were required. The cellophane bag was then removed. After standing overnight, the tube was again centrifuged until the precipitate was well packed. The supernatant was discarded and the precipitate taken up in 0.9 per cent NaCl solution. The protein concentration, consisting almost entirely of gamma globulin,[4] was estimated by the turbidometric method of Ayer, Dailey, and Fremont-Smith.[5]

Care should be taken to approximate the end point as closely as possible. If, however, the end point should be passed slightly, an amount of serum exactly equal to the excess of ammonium sulfate added should be pipetted into the centrifuge tube. The serum added is then mixed well with the supernatant and the tube warmed to 35 to 40° C. for thirty minutes, following which it is permitted to stand overnight and treated in the usual fashion.

5. The value for beta plus gamma globulin was obtained by subtracting the value for albumin plus alpha globulin from total protein values (Step 1 minus Step 2).
6. Beta globulin was estimated by subtracting the value obtained for gamma globulin (Step 4) from the value obtained for beta plus gamma globulin (Step 5).
7. Alpha globulin concentration was obtained by subtracting the value for albumin (Step 3) from that of albumin plus alpha globulin (Step 2).

These determinations can be accomplished in three hours of actual work. However, since the gamma globulin precipitation requires standing overnight twelve to eighteen hours are needed from the time the samples are received. It is preferable that the determinations be carried out in duplicate. For the complete analyses in duplicate 6.5 ml. of serum are required.

RESULTS AND DISCUSSION

A consideration of the method outlined will show that its operation fundamentally depends upon the assumption that the "albumin" found by the Howe method of sodium sulfate precipitation actually is equivalent to the electro-

phoretically determined true albumin plus alpha globulin, and that the "globulin" of the Howe method actually is equivalent to beta plus gamma globulin. This assumption was originally based upon a review of the older papers in which sodium sulfate and electrophoretic fractionation were compared.[6, 7] While the present study was in progress, Petermann, Young, Hoagness, and Rhoads[8] also observed the coincidence in values between the Howe "albumin," on one hand, and the sum of the electrophoretic values for albumin and alpha globulin on the other.

The data obtained in the course of this study appear to justify this assumption. Table I lists the values obtained for various serum components by the chemical and the electrophoretic method in human subjects. In both Table I and Table II good correspondence is shown between the Howe "albumin" (albumin plus alpha globulin obtained by the chemical method) and the electrophoretic values for albumin plus alpha globulin.

No difficulty was encountered in obtaining duplicate checks of the values for total protein, albumin plus alpha globulin, beta plus gamma globulin, and gamma globulin. However, we agree with Petermann and co-workers[8] in emphasizing that to obtain reliable and reproducible results for albumin, the most meticulous care must be used in the preparation of the reagents, in avoiding evaporation, and in maintaining all components of the system at 0° C. throughout the precipitation and filtration process. Because of the delicacy of the Pillemer and Hutchinson method,[3] consideration is being given to the possibility of substituting the albumin method suggested by Chow[9] which he has shown also to give values for albumin which approximate electrophoretic values.

In Table II we have compared all average values obtained by the chemical method with those obtained by electrophoresis. It can be seen that the average difference between the two methods does not exceed 10 per cent for any fraction. Electrophoretic values depend on the particular buffer used and some fractions may vary as much as 60 per cent in successive determinations on the same sample with different buffers. The agreement between the electrophoretic and precipitation methods appears to be satisfactory.

The agreement between individual electrophoretic and chemical results* was somewhat poorer for the animal sera than for the human. It appears likely that this resulted from the necessity of removing fibrinogen by the use of clotting thrombin before the samples could be analyzed.[10]

In addition to its general availability, the chemical method has one advantage not shared by the Tiselius procedure. Since the biuret method employed for the individual determinations estimated protein alone, other substances of lipoid or carbohydrate nature which migrate with protein fractions are not measured. In the Tiselius apparatus, values for such protein-bound substances are included in calculating the percentage composition of the various fractions. On the other hand, the Tiselius method provides a direct check on the purity of the individual fractions in the schlieren diagram; and it also permits determination of subfractions of the major globulin fractions.

*We have been requested to withhold individual electrophoretic analyses of the animal samples as these form a portion of studies to be published separately.

TABLE I. COMPARISON OF PROTEIN FRACTIONATION VALUES OBTAINED BY ELECTROPHORESIS AND CHEMICAL PRECIPITATION IN HUMAN SERA

SERUM	DIAGNOSIS	FRACTIONATION METHOD	TOTAL PROTEIN (GM. %)	ALBUMIN (GM. %)	GLOBULIN (GM. %)		
					ALPHA	BETA	GAMMA
1	Normal	Chemical	7.2	4.1	1.4	0.41	1.4
		Electrophoresis	7.3	3.9	1.3	0.77	1.3
2	Hypogammaglobulinemia with lymphopenia and lymphatic atrophy and bronchiectasis	Chemical	3.5	2.0	0.9	0.47	0.13
		Electrophoresis	3.6	2.1	0.9	0.41	0.15
3	Chronic glomerulonephritis, nephrotic phase	Chemical	4.0	0.8	1.2	1.7	0.33
		Electrophoresis	4.0	0.8	1.6	1.4	0 25
4	Sarcoidosis?	Chemical	9.2	2.7	1.1	1.2	4.2
		Electrophoresis	9.1	3.1	0.7	1.1	4.2

TABLE II. COMPARISON OF PROTEIN FRACTIONATION BY CHEMICAL AND ELECTROPHORETIC METHODS*

SERA	ALBUMIN	ALBUMIN PLUS ALPHA GLOBULIN	ALPHA GLOBULIN	BETA PLUS GAMMA GLOBULIN	BETA GLOBULIN	GAMMA GLOBULIN
Human†	0.97	1.01	1.02	1.03	1.03	1.03
Farm animals‡	0.95	0.98	1.07	1.08	1.10	1.05

*The results are expressed as the ratio of the protein fraction obtained by chemical methods to the protein fraction by electrophoresis.
†Results from one normal and three abnormal sera.
‡Results from three sheep, one horse, and one porcine sera.

SUMMARY AND CONCLUSION

A precipitation method for the fractionation of sera is described. This method gives values for albumin and alpha, beta, and gamma globulin which show good agreement with those obtained by electrophoretic analyses.

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SODIUM AND POTASSIUM DETERMINATIONS IN HEALTH AND DISEASE

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THE data in this report will be presented in three segments: (1) the concentration of sodium and potassium in the sera of 107 normal adults, (2) the results of various recovery experiments, and (3) several cases demonstrating the clinical application of sodium and potassium determinations. The flame photometer[1, 2] offered a rapid means of accurately determining the concentrations of these ions.

APPARATUS

The flame photometer used in these observations was the Perkin-Elmer instrument, Model 18. The recommended technique was followed.[2] The burner was fed natural gas from the city mains via the recommended constant pressure adjustment. The air pressure leading to the atomizer was maintained at 14 pounds. Two adjustments were necessary in relation to these features of the instrument before constant reproducible readings were obtained. By trial and error method the aperture of the jet giving the most constant readings was found to be 0.8 millimeter. This 0.8 mm. aperture was made by soldering a thin sheet of brass over the opening of the larger jet supplied and gradually reaming out a hole that would produce a flame of maximum heat without "feathering" (that is, loss of the small blue cones above the burner grill). Second, a moisture trap was placed in the air pressure line to keep chemically contaminated moisture away from the flame.

It is understood that improved instruments which circumvent these difficulties will be available in the future.[3]

NORMAL SERUM SODIUM AND POTASSIUM VALUES

Serum Na and K concentrations were determined on 107 normal, ambulatory adults (blood donors). Most of these subjects had been at rest more than twenty minutes prior to the collection of samples. The samples were taken at the end of the blood collections in the blood bank and consisted of the last column of blood in the collecting tube. This blood was allowed to drain by gravity into a test tube after withdrawal of the needle from the vacuum bottle. Test tubes previously were rinsed scrupulously with sodium- and potassium-free distilled water. The clot was allowed to contract and the serum was obtained by centrifuging.

The actual analysis for sodium or potassium was simple. For Na, the serum was diluted 1:100 with distilled water. The instrument was set at zero

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with the same distilled water and at 100 with a standard sodium solution. The standard used contained 50 ppm of sodium in the form of pure NaCl. The sodium concentration was obtained by the equation $C_u = \frac{C_s\ R_u}{R_s} \times 100$ where C_u is the concentration of the unknown, C_s the concentration of the standard, R_u the reading of the unknown, and R_s the reading of the standard. The equation is based on a linear relationship of dilutions of the 50 ppm standard with the instrument used (see Table VI). The K concentration determination was similar. The serum was diluted 1:10 with distilled water. The standard KCl solution contained 100 ppm of K.

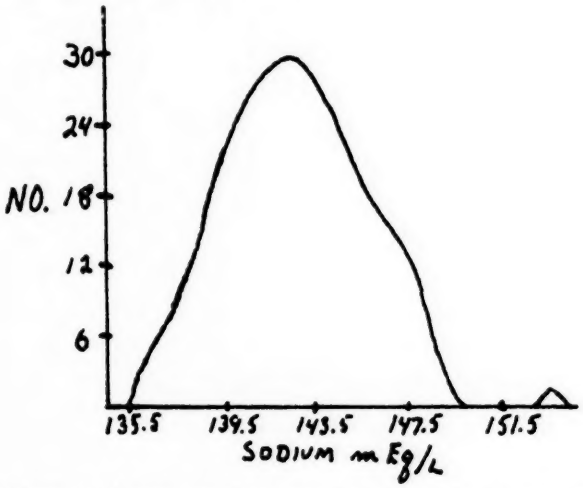

Fig. 1.—Mean, 144.0 meq. per liter; standard deviation, 3.6 meq. per liter.

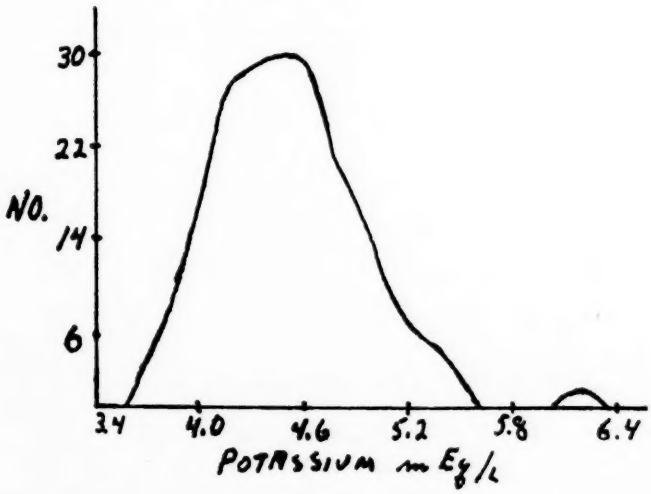

Fig. 2.—Mean, 4.52 meq. per liter; standard deviation, .45 meq. per liter.

Tables I and II contain the frequency distribution for the sodium and potassium determinations respectively. For the serum sodium values the following figures in milliequivalents per liter were determined: range 135.5 to

153.2; mean 144.0; standard deviation (σ) 3.6; coefficient of variation, 2.5 per cent. The potassium values in milliequivalent per liter were: range 3.6 × 6.2; mean 4.52; standard deviation 0.45; coefficient of variation 9.9 per cent. For comparison Table III shows the normal ranges as reported by other methods.

TABLE I. FREQUENCY DISTRIBUTION; NORMAL SERUM SODIUM DETERMINATIONS

NA (MEQ./L)	NO.
135.5	1
137.5	9
139.5	22
141.5	29
143.5	30
147.5	13
149.5	1
151.5	0
153.5	2

TABLE II. FREQUENCY DISTRIBUTION; NORMAL SERUM POTASSIUM DETERMINATIONS

K (MEQ./L)	NO.
3.6	1
3.9	11
4.2	28
4.5	30
4.8	20
5.1	9
5.4	5
5.7	0
6.0	1
6.2	1

TABLE III. NORMAL RANGES AS REPORTED BY OTHER METHODS

AUTHOR	NORMAL SERUM DETERMINATIONS NO.	RANGE		METHOD
		NA (MEQ./L)	K (MEQ./L)	
Kramer and Tisdall[4]	10		4.8 -5.1	Unashed serum
	16		4.6 -5.4	Ashed serum
Meyers and Short[5]	4		3.6 -4.6	Ash method
Atchley, and others[6]	6		3.1 -5.2	Precipitation method
	6	111.2-146.5		Gravimetric method
Rourke[7]	14	143.9-150.8		Iodometric method
Kramer and Tisdall[8]	17	140.4-152.1		Direct gravimetric
		145.0-150.0		Ashed
Consolazio and Talbott[9]	37	135.0-143.0	3.88-5.59	Electrodialysis
Consolazio and Talbott[10]	11		3.95-7.11	Titration
Sunderman[11]	10	133.6-137.3		Gravimetric method
	16		3.8 -4.3	Titrimetric
Hald[12]	8	134.2-141.0	3.1 -5.3	Dry ash method
Leva and Guest[13]	3	139.0-142.1	4.3 -5.0	
Albanese and Wagner[14]	6		3.7 -5.6	Colorimetric

STANDARDIZATION OF ANALYTICAL PROCEDURES

Table IV contains data on estimations of sodium concentrations. Sixteen separate samples of serum from normal adults were divided into four groups

of four each. The following amounts of sodium in milliequivalent per liter were added: 2.17 to the first group, 10.85 to the second, 21.7 to the third, and 43.4 to the fourth. The fourth column in the table gives the expected theoretic amounts to be recovered, and the fifth column gives the actual quantities determined by the flame photometer. The deviations observed are given in Column six.

TABLE IV. RECOVERY OF SODIUM ADDED TO NORMAL HUMAN SERUM

SAMPLE	ORIGINAL VALUE (MEQ./L.)	NA ADDED (MEQ./L.)	THEORETIC NA CONCENTRATION (MEQ./L.)	RECOVERY VALUE (MEQ./L.)	DEVIATION (MEQ./L.)
1	141.3	2.17	143.47	144.34	+0.87
2	145.7	2.17	147.87	149.34	+1.47
3	147.8	2.17	149.97	150.00	+0.03
4	150.9	2.17	153.07	150.00	−3.07
5	146.7	10.85	157.55	156.10	−1.45
6	143.0	10.85	153.85	153.90	+0.05
7	146.1	10.85	156.95	155.60	−1.35
8	144.6	10.85	155.45	157.80	+2.35
9	143.9	21.70	165.60	162.60	−3.00
10	146.1	21.70	167.80	166.90	−0.90
11	146.1	21.70	167.80	164.70	−3.10
12	145.7	21.70	167.40	166.10	−1.30
13	145.7	43.40	189.10	190.20	+1.10
14	145.2	43.40	188.60	186.10	−2.50
15	148.6	43.40	192.00	184.40	−7.60
16	149.3	43.40	192.70	193.00	+0.30

TABLE V. RECOVERY OF POTASSIUM ADDED TO NORMAL HUMAN SERUM

SAMPLE	ORIGINAL VALUE (MEQ./L.)	K ADDED (MEQ./L.)	THEORETIC K CONCENTRATION (MEQ./L.)	RECOVERY VALUE (MEQ./L.)	DEVIATION (MEQ./L.)
1	4.90	1.92	6.82	7.00	+0.18
2	5.30	1.92	7.22	6.90	−0.32
3	5.00	1.92	6.92	7.50	+0.52
4	5.40	1.92	7.32	7.40	+0.08
5	5.00	3.84	8.84	9.20	+0.36
6	6.20	3.84	10.04	10.50	+0.46
7	4.60	3.84	8.44	8.70	+0.26
8	4.70	3.84	8.54	8.20	−0.34
9	4.90	7.68	12.58	12.30	−0.28
10	4.70	7.68	12.38	12.90	+0.52
11	6.30	7.68	13.98	13.50	−0.48
12	5.30	7.68	12.98	12.70	−0.28
13	4.60	11.52	16.12	15.80	−0.32
14	5.20	11.52	16.72	16.10	−0.62
15	4.50	11.52	16.02	15.60	−0.42
16	4.70	11.52	16.22	16.10	−0.12

Table V has identical information concerning the sixteen samples with reference to the potassium concentration in milliequivalent per liter.

The deviations observed in these determinations are considered to be within the variations of standard clinical laboratory tests and indicate a satisfactory clinical applicability of the procedure.

In the next group of observations variations in the concentration of the standard solutions were made and recovery was tested. With Na (Table VI)

a standard of 50 ppm was used. The instrument was set at 100 with this solution and dilutions of the solution were tested for recovery. Three different groups were tested. Table VII has the same type of information with K standard solutions containing 100 parts per million. The observations in these groups indicate a degree of recovery which is quite satisfactory for clinical use.

TABLE VI. PROPORTIONALITY OF PHOTOMETER READINGS TO VARIED CONCENTRATIONS OF SODIUM

GROUP	CONCENTRATION BY WEIGHT (PPM)	READING ON PHOTOMETER	RECOVERY (PPM)	DEVIATION (PPM)
I	50	100.0	50.0	0.0
	40	83.0	41.5	1.5
	30	64.8	32.4	2.4
	20	40.1	20.0	0.0
	10	22.3	11.1	1.1
II	50	100.0	50.0	0.0
	40	81.2	40.6	0.6
	30	63.2	31.6	1.6
	25	52.3	26.1	1.6
	20	43.0	21.5	1.5
	15	32.8	16.4	1.4
	10	20.3	10.1	0.1
III	50	100.0	50.0	0.0
	40	81.2	40.6	0.6
	30	60.3	30.1	0.1
	20	40.2	20.1	0.1
	10	20.6	10.3	0.3

TABLE VII. PROPORTIONALITY OF PHOTOMETER READINGS TO VARIED CONCENTRATIONS OF POTASSIUM

GROUP	CONCENTRATION BY WEIGHT (PPM)	READING ON PHOTOMETER	RECOVERY (PPM)	DEVIATION (PPM)
I	100	100.0	100.0	0.0
	75	75.2	75.2	0.2
	50	50.0	50.0	0.0
	25	24.8	24.8	0.2
	20	20.3	20.3	0.3
	15	15.1	15.1	0.1
	10	10.0	10.0	0.0
II	100	100.0	100.0	0.0
	75	74.7	74.7	0.3
	50	52.2	52.2	0.2
	25	24.9	24.9	0.1
	15	15.0	15.0	0.0
	5	4.9	4.9	0.1
III	100	100.0	100.0	0.0
	75	74.9	74.9	0.1
	50	50.3	50.3	0.3
	25	24.8	24.8	0.2
	20	20.0	20.0	0.0
	15	15.1	15.1	0.1
	10	10.1	10.1	0.1

The influence of the dilution of the serum on the concentration values was tested for Na and is related in Table VIII. Greater variations were observed in this group of observations than in the former group. The 1:100 dilution seemed preferable because of higher and more stable readings.

TABLE VIII. EFFECT OF DILUTION OF SERUM ON THE PROPORTIONALITY OF PHOTOMETER READINGS TO CONCENTRATIONS OF SODIUM

SERUM	1:100 DILUTION		0.5:100 DILUTION		DEVIATION
	READING	MEQ./L.	READING	MEQ./L.	MEQ./L.
I	63.2	137.4	31.1	135.2	2.2
II	74.0	160.9	37.8	164.3	3.4
III	70.2	152.6	35.4	153.9	1.3
IV	63.4	137.8	30.1	130.9	6.9
V	67.3	146.3	33.7	146.5	0.2

Next, duplicate samples were tested for their potassium concentration. The results, given in Table IX, were quite satisfactory.

CLINICAL APPLICATION

The pertinent features of several cases are presented as illustrations of the applicability of this procedure to routine clinical use.

TABLE IX. CONSISTENCY OF DUPLICATE RESULTS

SAMPLE	READING	MEQ./L.	DEVIATION (MEQ./L.)
1A	13.1	3.3	
1B	13.3	3.4	0.1
2A	16.0	4.1	
2B	16.2	4.2	0.1
3A	17.5	4.5	
3B	17.1	4.4	0.1
4A	14.9	3.8	
4B	14.8	3.8	0.0
5A	16.7	4.3	
5B	16.7	4.3	0.0
6A	12.2	3.1	
6B	12.3	3.2	0.1
7A	13.3	3.4	
7B	13.6	3.5	0.1
8A	19.0	4.9	
8B	19.0	4.9	0.0
9A	17.4	4.5	
9B	17.2	4.4	0.1

TABLE X

DAY	BLOOD UREA (MG./100 C.C.)	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)	URINE VOLUME (C.C.)	URINE NA (GM.)	URINE K (GM.)
2	107	144	7.2	12		
3	186			30		
4	200	146	6.4	74		
5	245	137	5.1	39		
6	338			100		
7	458	135	5.4	124		
8	475	130	5.9	206		
9				540	1.4	0.16
10	406	135	4.6	1480	2.86	0.166
11	435			2300	4.59	0.62
12	458	132	5.0	6670	16.68	2.35
13	329	138	4.4	8770	19.4	2.2
14	279	129	5.1	6840	19.35	1.85
15	169	142	4.3	6425		
16	85.3			5950	8.45	0.67
17	76	145	4.4	3660		
18	64			6685		
19	53					

Case 1.—The patient was D negative (Rh negative) and had been immunized to the D antigen by two pregnancies. After the second delivery she received 380 c.c. of D positive blood. A severe hemolytic reaction occurred and was followed by severe renal insufficiency (oliguria, heme casts, azotemia, hypertension, etc.). Hyponatremia developed but was prevented from becoming extreme by $NaHCO_3$ therapy. The K concentration was elevated at first but dropped to normal levels later. Table X contains the data related to Na and K.

The average $NaHCO_3$ intake per day prior to the diuresis was 4 grams. During the diuresis attempts were made to replace the Na and K lost on a gram per gram basis.

Case 2.—The patient experienced extreme post-partum bleeding which necessitated a hysterectomy. Hypotension existed for fifty-one hours and was followed by renal insufficiency. A "force fluids" regime led to water intoxication. When seen eight days after the hypotension an alarmingly high serum potassium concentration was observed. Twenty grams of $NaHCO_3$ were quickly given by stomach tube, followed by 130 c.c. of 5 per cent NaCl solution by vein in two doses. The K value receded from the danger level and the Na concentration was elevated. Table XI presents data on this case.

TABLE XI

DAY	BLOOD UREA (MG./100 C.C.)	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)	URINE VOLUME (C.C.)	URINE NA (GM.)	URINE K (GM.)
1				170		
2	146			375		
3				300		
4	236			515		
5				955		
6	221			1070		
7	245			910+		
8	299	118	10.5	1200	1.44	0.93
9	291	125	6.1	2200	1.6	
10				3000		
11	355	137	5.0	3700		
12	370	137	4.1	5000	9.7	3.3
13	330	153	2.3	4300		

This patient died on the fourteenth day and an autopsy demonstrated severe and generalized brain damage. The kidneys were recovering satisfactorily at the time of death. The patient was in fluid balance except for a lowered K concentration which was being corrected.

Case 3.—This patient had a K deficit following a partial gastrectomy with posterior gastrojejunostomy. There was a poorly functioning stoma and vomiting was prolonged. He had received dextrose solution by vein daily. He was treated with KCl.* The diag-

TABLE XII

POSTOPERATIVE DAY	SERUM K (MEQ./L.)	SERUM NA (MEQ./L.)	THERAPY AND COURSE
12	3		Muscular weakness, hyperactive reflexes, irrational
14	3.6	146.0	Clinical improvement after 40 c.c. 15% KCl I.V.
15	3.8		25 c.c. 15% KCl I.V.
16	3.9		20 c.c. 15% KCl I.V.
17	4.2		Stoma functioning, considered in fluid balance
19	4.3		
20	4.5		
21	4.5		
23	4.6		Four days previous to hospital discharge.

*By Dr. Louis Kregel (Resident in Surgery) and Dr. Carl Moyer (Consultant).

noses* were (1) extracellular volume deficit with alkalosis and relative water excess and (2) relatively severe potassium deficit. The K values are shown in Table XII.

Case 4.—This was a case of fluid imbalance following a partial gastrectomy with Polya-Balfour anastomosis. On the seventh post-operative day a diagnosis of extracellular fluid deficit with a relative water excess was made by the surgical staff. Frequent serum Na and K determinations along with the clinical signs facilitated the handling of this case with hypertonic NaCl solution. The data are shown in Table XIII.

TABLE XIII

POSTOPERATIVE DAY	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)	THERAPY AND COURSE
6	123	7.7	
7	129.0	6.6	200 c.c. 5% NaCl I.V.
8	137	5.6	400 c.c. 5% NaCl I.V.
10	152	5.1	
12	137	5.6	Clinically in fluid balance

TABLE XIV

HOSPITAL DAY	SERUM NA	SERUM K (MEQ./L.)	24 HOUR GASTRIC ASPIRATIONS (GM.)		24 HOUR URINE (GM.)	
			NA	K	NA	K
12	149.6	3.6				
14			.293	.039	.975	.510
18	156.5	2.7	1.287	.586	.881	.593
19			.616	.158	1.350	.549
20			.408	.094	1.996	.740
21			1.105	.119	2.130	1.360
24			1.849	.591	2.820	1.670
25			1.540	.764	1.986	1.221
26			1.330	.683	2.073	1.170
38	146	4.2				

TABLE XV

DAY	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)
1	115.7	6.7
2	126.5	5.4
3	135.0	

TABLE XVI

DAY	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)
6	148	4.2
13	135	5.1
15	141	4.1
18	139	4.4

Case 5.—This case was one of chronic pyloric obstruction secondary to duodenal ulcer. There were Na and K determinations of the serum, gastric contents, and urine. There was a persistently low serum K concentration without any clinical signs of K deficit. After three weeks a gastroenterostomy was performed; recovery was uneventful. Table XIV presents the data.

*Made by Dr. Moyer.

Case 6.—A patient with Addison's disease was studied during the terminal crisis. The values of K and Na during the last three days are shown in Table XV.

Case 7.—A patient with adenocarcinoma of the stomach was studied during the postoperative period. The normal values obtained are shown in Table XVI.

SUMMARY

1. A series of observations of the concentration of Na and K in body fluids has been made using the flame photometer technique.

2. Determination of sodium and potassium in the blood sera of 107 normal blood donors was made. The average for sodium was 144.0 meq. per liter, with a range of 135.5 to 153.2 meq. per liter. The average for potassium was 4.52 meq. per liter, with a range of 3.6 to 6.2 meq. per liter.

3. Recovery experiments, duplicate determinations, and distribution curves of normal values indicate satisfactory accuracy for clinical use.

4. The presence of sodium, and also potassium, in the blood sera of several patients with various diseases was determined.

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CHANGES IN THE BONE MARROW IN MEGALOBLASTIC ANEMIAS OF INFANCY BEFORE AND AFTER FOLIC ACID THERAPY

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UNDER the term megaloblastic anemia of infancy we have previously described an anemia characterized by changes in the blood and bone marrow resembling those of Addisonian pernicious anemia and by a specific response to folic acid.[1-3] The present report deals with the results of serial studies of the bone marrow which were undertaken in patients with this type of anemia for the purpose of elucidating the mechanisms underlying the development of the megaloblastic dysplasia and its disappearance under specific therapy.

MATERIAL AND METHODS

Our material consisted of fifteen patients* with megaloblastic anemia observed during a period of twenty-one months at the Children's Hospital of Michigan. All the patients were white infants ranging in age from five weeks to one year. The clinical features were not characteristic. Usually there were signs of infection, and in three instances scurvy was noted. Eleven of the fifteen patients had histamine-resistant achlorhydria at the time therapy was begun. Macrocytic anemia of varying degree was present in all cases. The hemoglobin levels on admission ranged from 1.7 to 8.3 Gm. per 100 c.c. (Table I). The percentage of reticulocytes was variable and in some instances moderately elevated even before therapy. Prior to folic acid therapy six patients received transfusions of whole blood and two others, transfusions of saline suspended washed red cells. Folic acid† was administered by intramuscular injection in all but two instances, the daily dosage varying from 10 to 100 mg. and the total dosage, from 50 to 900 milligrams. The average course of treatment consisted of two daily injections of 10 mg. over a period of one week. To two patients folic acid (*Lactobacillus casei* factor) was given by mouth in daily doses of 5 mg. for ten-day periods. Since it was evident that there was no appreciable difference in the response between patients receiving lower and higher dosage within the range given, no further reference to dosage will be made. In every case a characteristic increase in reticulocytes, up to 23 per cent, took place around the fifth day after the start of therapy, regardless of the initial degree of reticulocytosis, and hemoglobin and red cell counts rose significantly.

Daily complete blood counts and reticulocyte counts were made. Bone marrow was aspirated from the femur, and occasionally from the upper end of the tibia. In each instance at least one marrow sample before and after treatment was taken and in some instances as many as six serial examinations were made. A measured amount of marrow was withdrawn, usually between 1.0 and 2.0 c.c., although occasionally more or less marrow was accidentally obtained. The samples were placed in paraffin tubes containing heparin. One cubic centimeter was transferred into a Wintrobe tube, centrifuged for five minutes at 3,000 r.p.m., and the height of the various layers was recorded. The myeloid-erythroid

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*Clinical data on nine of these patients were included in our earlier reports.[2,3]

†Synthetic folic acid was obtained through the courtesy of Lederle Laboratories, Inc., Pearl River, N. Y.

TABLE I. BLOOD, BONE MARROW, AND GASTRIC JUICE BEFORE THERAPY

CASE	INITIAL BLOOD FINDINGS			INITIAL MARROW FINDINGS			PRESENCE OF FREE HCL AFTER HISTAMINE
	HEMOGLOBIN (GM./100 C.C.)	R.B.C. (MIL./C.MM.)	RETICULOCYTES (%)	MARROW ASPIRATED (C.C.)	MYELOID-ERYTHROID LAYER (VOL. %)	ERYTHROID LAYER* (VOL. %)	
1	5.0	1.12	1.5	1.0	2.0	0.7	0
2	4.9	1.49	6.9	1.3	4.0	2.0	0
3	6.8†	2.52	0.9	2.25	20.0	9.0	+
4	8.0	2.25	3.1	1.5	30.0	11.1	0
5	3.5	1.12	0.1	?	2.0	1.0	0
6	1.7	0.30	0.3	0.7	0.91	0.006	0
7	5.8	2.01	0.7	1.54	10.5	1.5	0
8	8.3	2.53	2.7	1.1	12.0	3.2	+
9	6.6	2.05	2.7	2.2	5.0	1.8	0
10	7.7	2.75	5.8	2.0	26.0	13.9	+
11	5.0	1.33	1.3	2.25	2.0	0.8	0
12	6.2	2.40	1.0	2.25	5.0	1.0	‡
13	7.8	2.30	0.6	2.0	15.0	4.1	0
14	4.2	0.89	1.2	3.0	3.6	0.9	+
15	6.2	2.11	8.7	1.1	7.5	3.3	0

*Calculated from the proportion of erythroblasts obtained in a differential count of 500 nucleated cells.

†After transfusion.

‡Not examined.

layer was then thoroughly mixed on a paraffin-coated watchglass and films made from it were stained with May-Gruenwald-Giemsa. In each case a differential count of at least 500 nucleated cells was made, using the nomenclature of Downey[4] and Jones.[5] In addition, a count of 500 erythroblasts was made for each aspiration in eleven instances and in eight of these 100 mitoses in erythroblasts were classified for every sample.

QUALITATIVE CHANGES

Changes Before Therapy.—In six of the fifteen patients erythropoiesis was frankly megaloblastic.* As a rule the proportion of basophilic cells was high. This "shift to the left" in the maturation curve of the erythroid cells was paralleled by a relative increase in the number of mitoses encountered in basophilic cells. In examining many fields the total number of mitoses rarely appeared significantly increased and in some instances was clearly depressed, but actual counts were not made because the incidence of mitotic figures in only 500 cells would not have been significant. The mitotic figures were often distinctly pathologic. The chromosomes frequently appeared abnormally long and slender, the polarity of the mitotic spindle was often distorted, and aberrant chromosomes were common. Karyorrhexis and karyolysis were often pronounced. The granulocytes showed alterations in size, nuclear configuration, and cytoplasm indistinguishable from those seen in pernicious anemia.

In seven other patients classic megaloblasts were also present in fair numbers but did not constitute the majority of the erythroid cells. The predominant cell type, although closely resembling the megaloblast, failed to meet the strict criteria given by Jones[5] and others, yet these erythroblasts were distinctly abnormal and different from normoblasts. In the course of our studies it soon became evident that the current nomenclature of marrow cells which implies two types of erythropoiesis was inadequate for our purpose. It would

*For illustrations see our earlier communication.[3]

have forced us to place pathologic cells in the category of normoblasts simply because they did not correspond in every detail to megaloblasts. We therefore temporarily adopted the term "intermediate" for erythroid cells which we could not otherwise classify. We do not wish to imply that these cells represent a separate type of erythropoiesis but merely to indicate that *morphologically* they occupy a middle position between typical normoblasts and typical megaloblasts.

The cells of the intermediate type were large, usually as large as megaloblasts and always significantly larger than normoblasts. The ratio of cytoplasm to nucleus was often increased. The nucleus had an increased amount of acidophilic matter and the chromatin net was much finer and more regular than that of the normoblast but still slightly coarser than that of the classic megaloblast. The open structure of the nuclear chromatin persisted in the later, acidophilic stages of cell development, and in the more mature forms differentiation from megaloblasts became indeed almost impossible. As in typical megaloblasts, abnormal mitoses, irregular chromatolysis, and pronounced karyorrhexis were common features of these intermediate cells, and many of the nuclei, especially those of the later stages, had bizarre shapes and showed extreme lobulation.

We concluded that this intermediate type of erythropoiesis represents an early stage of megaloblastic transformation of the bone marrow. We were led to this view by the following observations:

1. The cells designated as intermediate resembled megaloblasts in all but the finest cytologic details.

2. At least a few, and often numerous, classic megaloblasts were always found when erythropoiesis was of the intermediate type but were never seen by us in normoblastic marrows. Conversely intermediate cells were nearly always present when megaloblasts predominated in the marrow.

3. The actual progression of megaloblastic transformation was observed in one patient (Fig. 1) where specific treatment was deliberately withheld for one month after the initial marrow examination had shown an intermediate type of erythrogenesis. At the end of that time the anemia had recurred and a second marrow specimen showed a considerable increase in classic megaloblasts.

4. In every case where intermediate erythroblasts predominated in the marrow the abnormalities seen in the granulocytes were the same as in cases with a frankly megaloblastic picture. We have not seen this type of leucopoiesis in other conditions.

5. A specific effect of folic acid was demonstrable in every instance and, as will be shown the response followed the same pattern as that of the first group (Fig. 7).

These observations suggested that the megaloblastic transformation of the bone marrow is a gradual and progressive change in which the appearance of typical megaloblasts and promegaloblasts is a relatively late event. Our findings seem to indicate that the process may be recognized in its earlier stages by the application of more liberal criteria in regard to erythrogenesis. Even

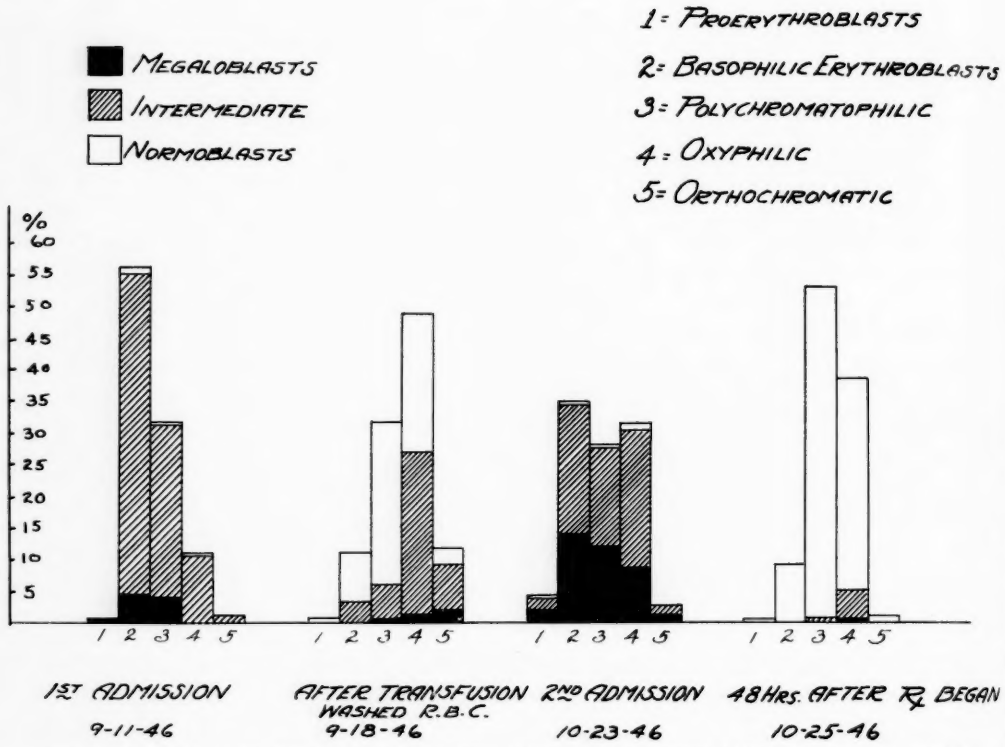

Fig. 1.—Case 13. The change in marrow from intermediate to megaloblastic type when specific therapy is withheld.

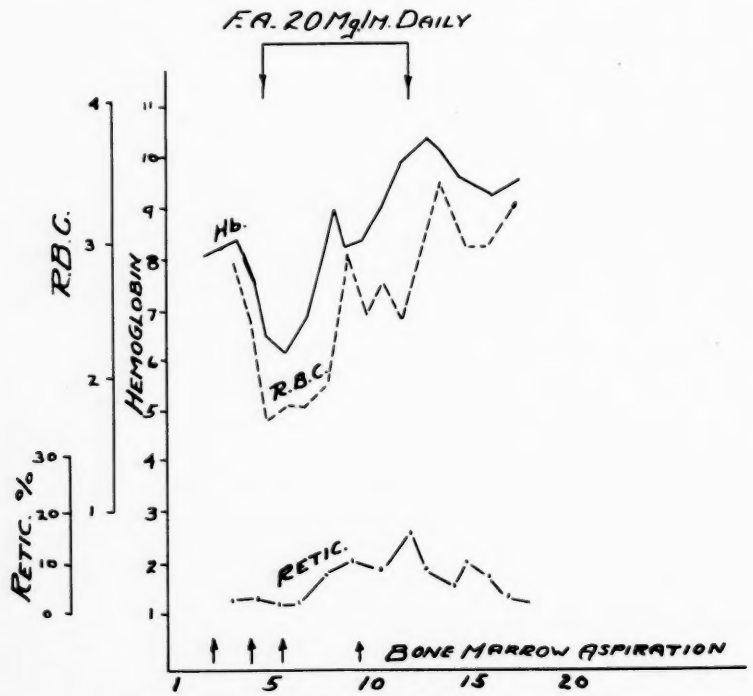

Fig. 2.—Case 4. Response of anemia to folic acid.

the pattern of erythropoiesis which we have referred to as intermediate appears to be preceded by constant and specific changes in the granulocytes which may be utilized for early diagnosis. The marrow of the remaining two patients in our group (Cases 4 and 7) showed only minimal deviations from the normoblastic pattern, but the normal myelocytes were largely replaced by "giant metamyelocytes" and similar pathologic granulocytes. In both instances the administration of folic acid was followed by reticulocyte responses of specific character and by subsequent rises in hemoglobin and red cell counts without further treatment (Fig. 2). Similar effects of folic acid were not seen in anemias other than those associated with megaloblastic bone marrow.[3]

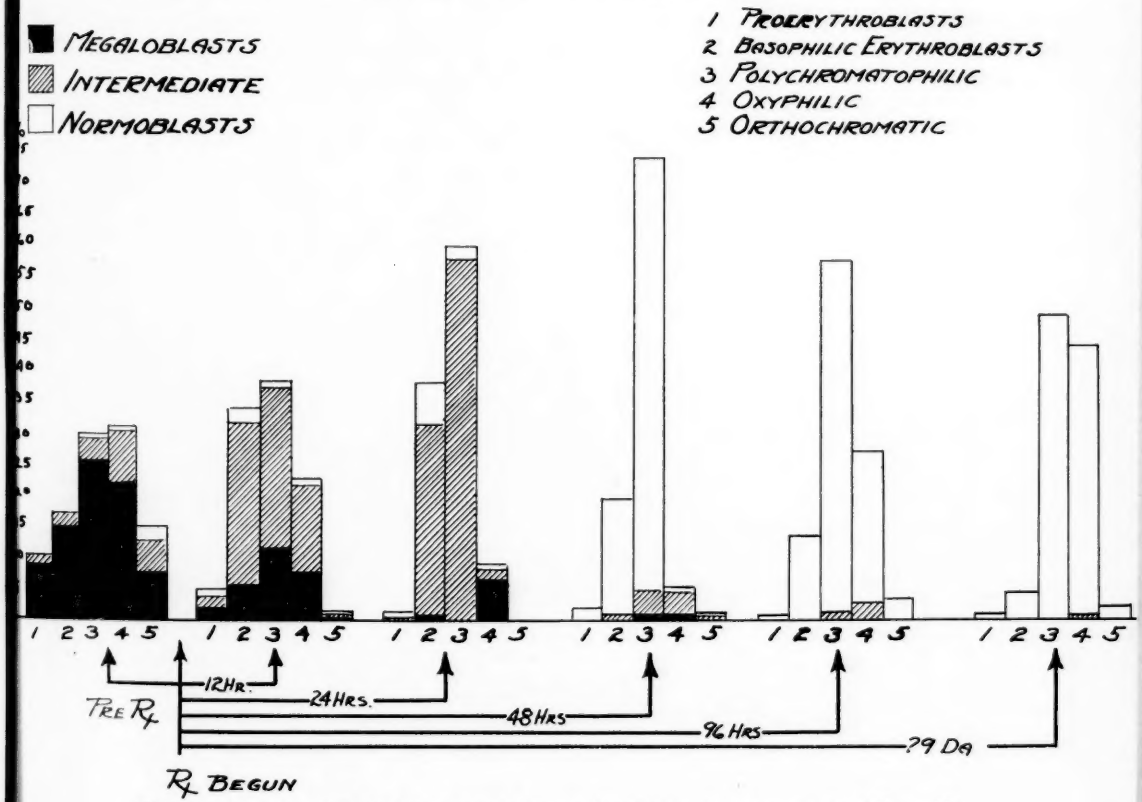

Fig. 3.—Case 8. Qualitative changes in erythropoiesis in response to folic acid.

Changes After Therapy.—In studying the marrow response to folic acid an attempt was made to obtain an over-all picture by taking samples at different intervals after the start of therapy. Therefore, the specimens taken from different patients were not always comparable as to the time when they were taken, but ranged from twelve hours to twenty-nine days, with most of the samples obtained in the first five days after treatment. Since it soon became evident that most of the significant changes took place during that period only a few observations were made in later stages.

Our only twelve-hour specimen came from a patient with a characteristic megaloblastic marrow pattern (Fig. 3). Before treatment almost no normo-

blastic cells were present. After twelve hours a significant change had occurred. Megaloblasts were still present but the vast majority of the cells had undergone a distinct change in appearance. Again, since they could not otherwise be classified, these cells were designated as intermediate in type. A small number of basophilic normoblasts was noted at this time.

At twenty-four hours the marrow of the same patient showed few megaloblasts and virtually all of these were acidophilic cells. There was a marked increase in basophilic and polychromatic erythroblasts which still did not appear entirely normal but closely resembled normoblasts. Simultaneously an appreciable number of basophilic normoblasts appeared. Numerous mitotic figures were seen.

At forty-eight hours erythropoiesis was typically normoblastic. There was a further marked increase in polychromatic erythroblasts.

At ninety-six hours a shift toward more mature cells had become evident. Almost 30 per cent of the erythroblasts were cells with oxyphilic cytoplasm.

Serial studies made on nine other patients showed a similar pattern (Figs. 4 to 7).* On the basis of these composite observations the progression of marrow changes in response to folic acid may be summarized as follows: The megaloblasts lose their characteristic structure, and in twelve to twenty-four hours the erythroblasts assume an appearance intermediate between that of megaloblasts and normoblasts. Typical normoblasts can already be seen in small numbers. Almost the only megaloblasts still recognizable at this time are late, oxyphilic cells, and these disappear at the end of thirty-six hours. Two days after treatment has begun the transformation is virtually complete and the marrow is normoblastic.

The change in the cellular characteristics is accompanied by an increase of basophilic and, slightly later, of polychromatic cells and by the appearance of numerous mitotic figures. In the following days there is a relative decrease in basophilic erythroblasts, a further increase in polychromatic cells, and a steady increase in oxyphilic cells so that the distribution of erythroid cells gradually approaches the normal.

The shifts in the maturation curves of the erythroblasts were rather closely paralleled by shifts in the distribution of the mitoses (Fig. 8). Before treatment there was usually a relative increase in mitosis of basophilic cells and a corresponding decrease of mitosis in more mature erythroblasts. Following therapy an impressive increase in the proportion of polychromatophilic cells in mitosis took place (Fig. 9). This suggested that the earlier disturbance had affected mitotic activity chiefly at the level of the polychromatophilic erythroblasts and that one effect of specific therapy was the resumption of mitotic division at the normal level of cellular maturation.

Our findings indicate that only the late, oxyphilic megaloblasts escape the effect of folic acid and are not transformed into normoblasts, presumably because they are past the stage in cellular development where multiplication occurs. These cells continue their maturation and probably leave the marrow

*To save space additional graphs showing the pattern in the four remaining cases have been omitted since they have essentially the same features as those shown.

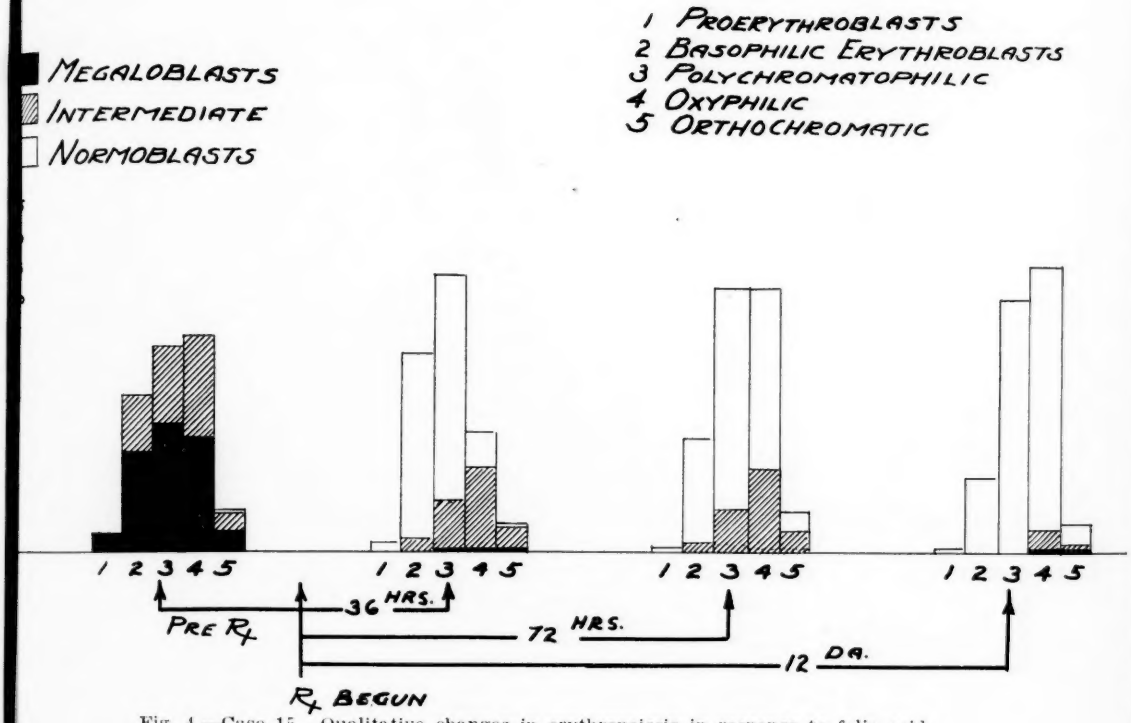

Fig. 4.—Case 15. Qualitative changes in erythropoiesis in response to folic acid.

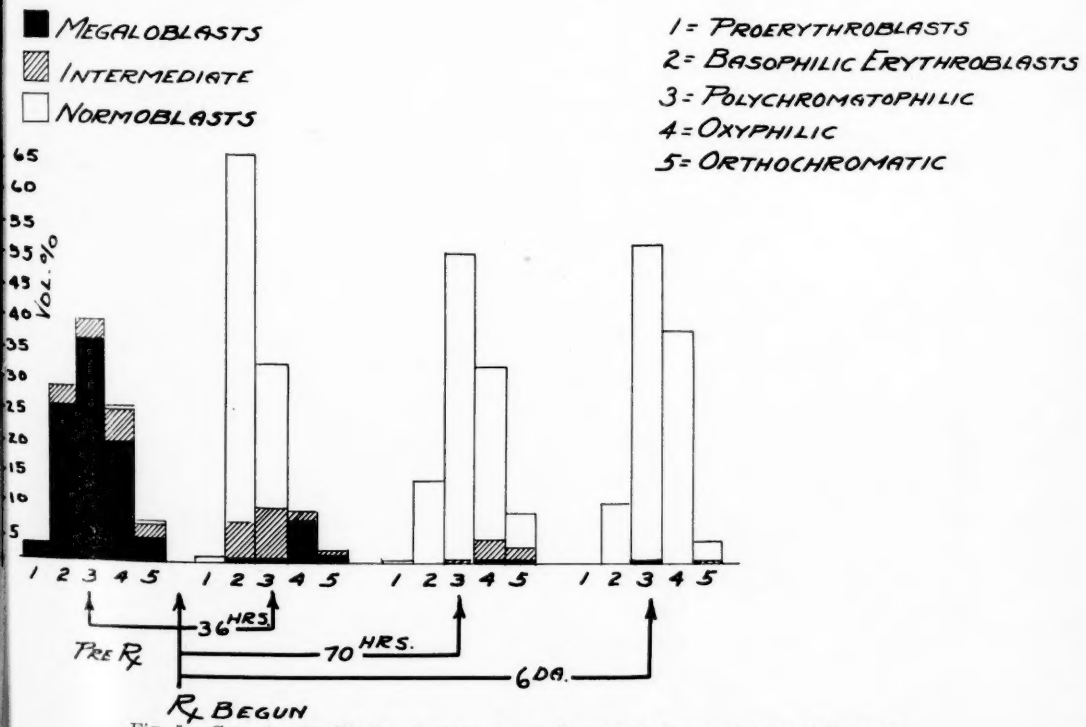

Fig. 5.—Case 6. Qualitative changes in erythropoiesis in response to folic acid.

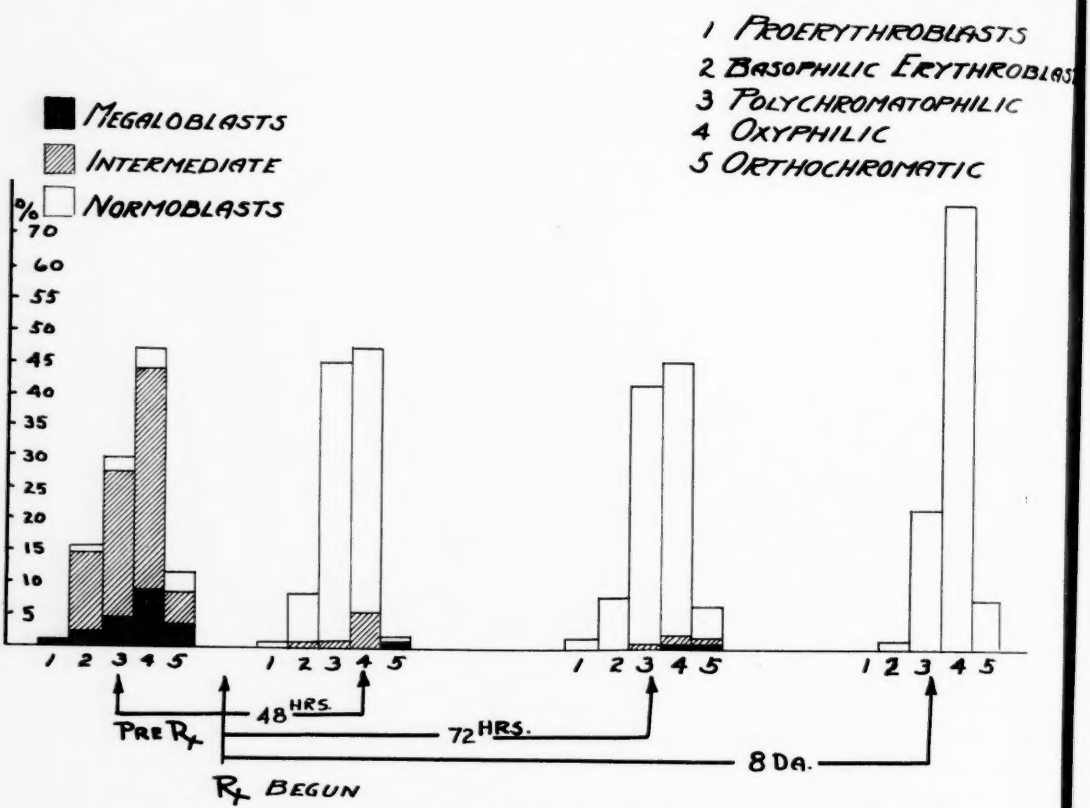

Fig. 6.—Case 9. Qualitative changes in erythropoiesis in response to folic acid.

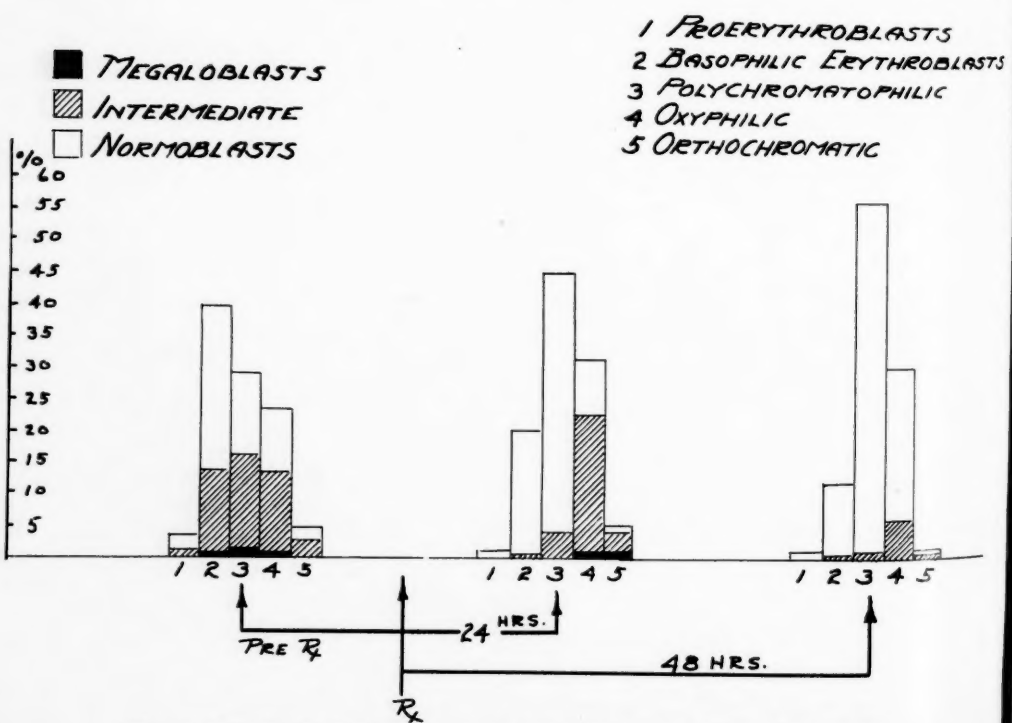

Fig. 7.—Case 7. Qualitative changes in erythropoiesis in response to folic acid.

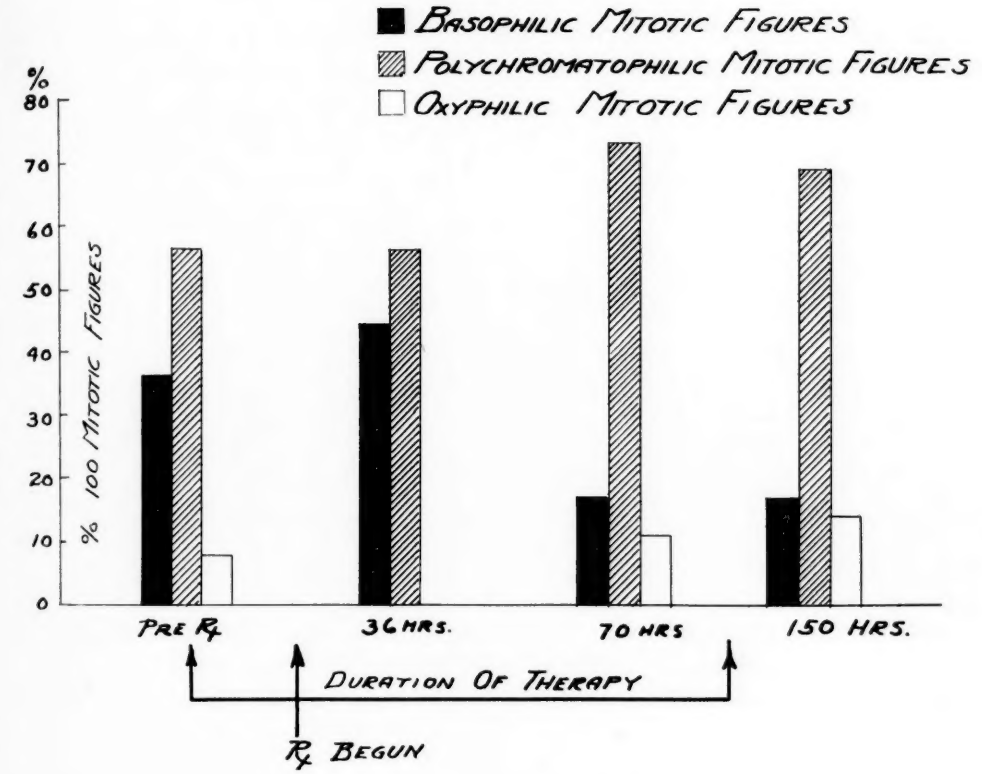

Fig. 8.—Case 6. Shift in erythroid mitosis after therapy.

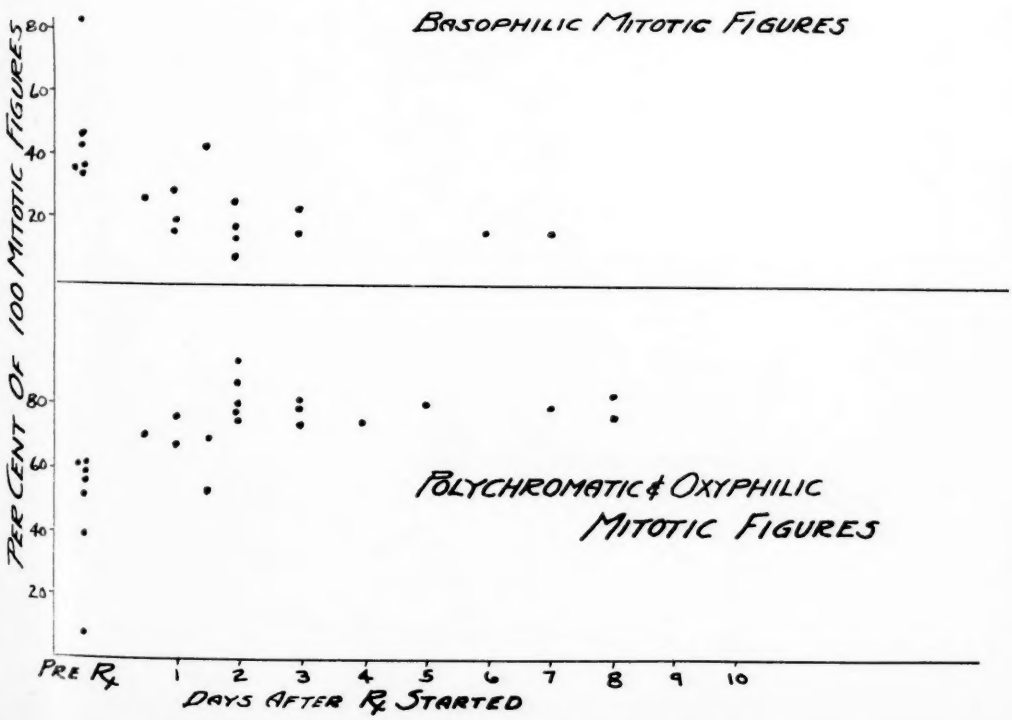

Fig. 9.—Trends of erythroid mitosis with therapy in eight cases.

as megalocytes. The younger megaloblasts, on the other hand, seem to be transformed almost quantitatively into normoblasts, even those already in the polychromatic stage. This is indicated not only by the enormous increase in polychromatic normoblasts early in the course of the response, an event which could hardly be explained by proliferation of the few residual normoblasts present before treatment, but more significantly by the virtually complete disappearance of megaloblasts in twenty-four to thirty-six hours. If the younger megaloblasts were not affected by the therapy but continued to mature as megaloblasts they should be demonstrable in proportionate numbers as oxyphilic megaloblasts in subsequent marrow samples. It appears likely, therefore, that as late as the polychromatic stage megaloblasts can utilize the hemopoietic principle and resume normal mitosis and normal morphologic characteristics.

Our observations on the evolution and regression of the megaloblastic pattern support neither the view that megaloblasts are normal constituents of the marrow nor the concept that these cells are genetically different from normal erythroblasts and immutably fixed in their characteristics by the nature of their ancestral cells. Instead we interpret our findings as evidence that in the absence of hemopoietic principle erythroblasts undergo a progressive aberration in structure as well as function which is reversible except in cells permanently incapable of mitosis. This concept in no way detracts from the diagnostic significance of the megaloblast.

QUANTITATIVE CHANGES

We are well aware of the limitations of measurements in the cellularity of the bone marrow.[6] Nevertheless it seemed worth while to investigate the volumetric pattern of the aspirated marrow in our patients to see if a constant pattern was demonstrable. In regard to the total cellularity before therapy this was not the case. Table I shows that the height of the myeloid-erythroid layer in the initial samples was too variable to be significant in itself. However,

TABLE II. CHANGES IN THE MYELOID-ERYTHROID LAYER WITH FOLIC ACID THERAPY

CASE	INITIAL M-E LAYER (VOL. %)	MYELOID-ERYTHROID LAYER AFTER START OF THERAPY (VOL. %)												
		HOUR				DAY								
		12	24	36	48	3	4	5	6	7	8	9	10	11	12
1	2.0							5.4							
2	4.0		9.0												
3	20.0						23.0								
4	30.0		13.0												
5	2.0										10.0				
6	0.1			21.5		14.0			22.0						
7	10.5		6.0		4.0	4.5									
8	12.0	9.0	30.5		40.0		35.3								
9	5.0				15.0	9.5					5.0				
10	26.0				5.5	8.0									9.5
11	2.0					10.0									
12	5.0								13.0						
13	15.0				6.0										
14	3.0											11.0			
15	7.5			26.0		16.0									13.5

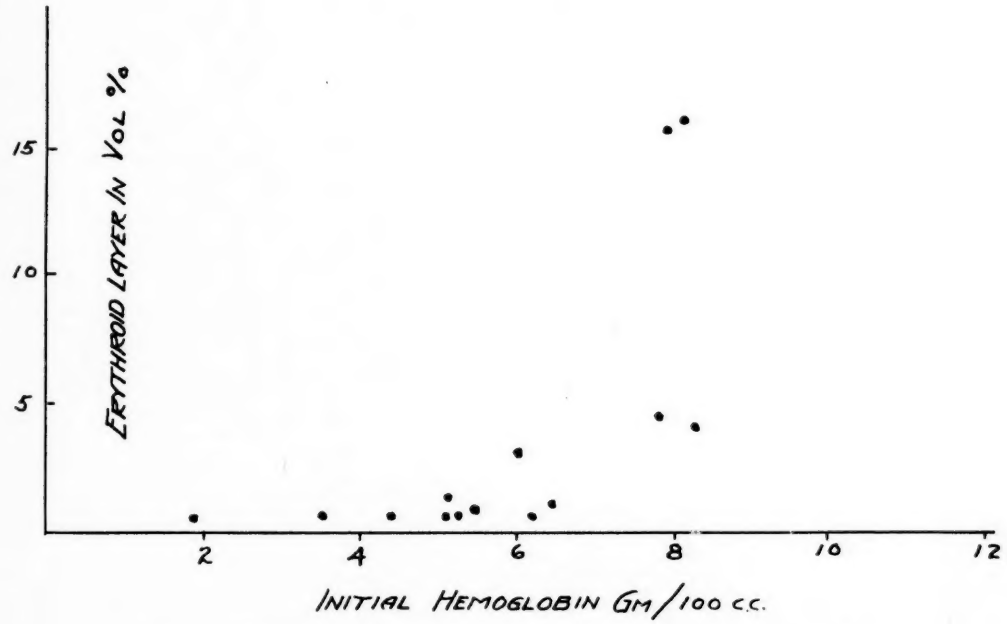

Fig. 10.—Relation of erythroid layer of initial marrow to initial hemoglobin.

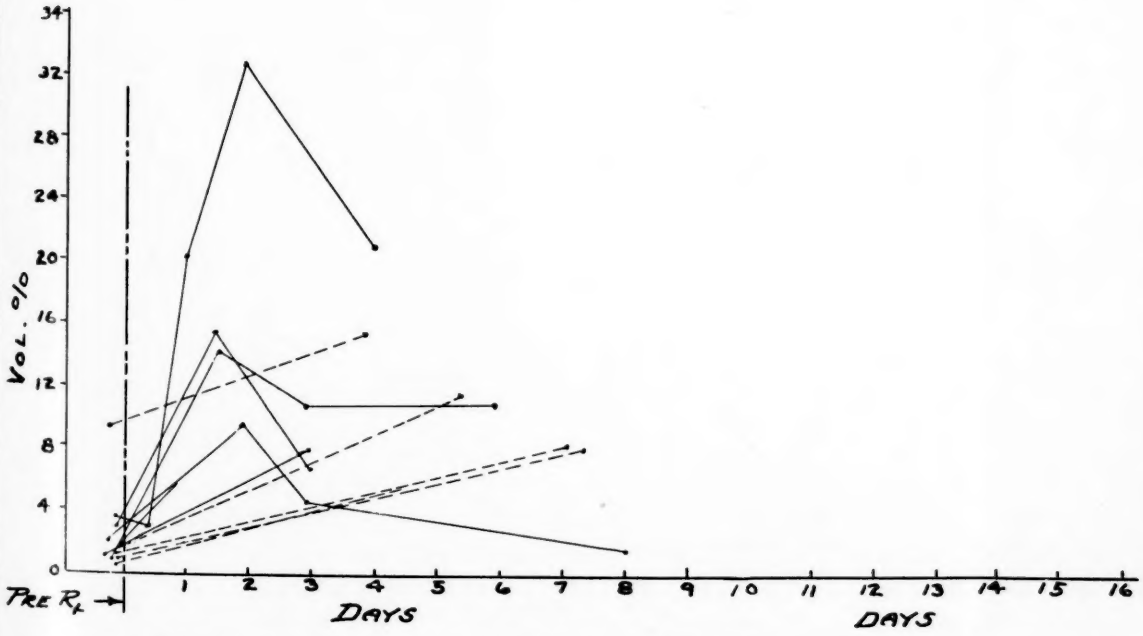

Fig. 11.—Increase in erythroid layer of buffy coat after folic acid therapy in twelve of fifteen cases. Dotted lines indicate only one marrow sample taken after treatment.

a substantial rise in the myeloid-erythroid volume was observed after therapy in eleven of the fifteen patients, suggesting a significant trend (Table II).

When the height of the myeloid-erythroid layer after centrifugation was recorded in volumes per cent and the proportion of erythroblasts determined by differential counts of 500 nucleated cells, the volume of erythroblasts could be expressed in per cent of the total sample, giving a sample measure of erythropoietic activity. Using these values we found rather good correlation between the initial erythroid cell volume in the marrow and the severity of the anemia (Fig. 10). Surprisingly enough, erythroid hyperplasia was the more marked the higher the hemoglobin; it was slight or absent in patients

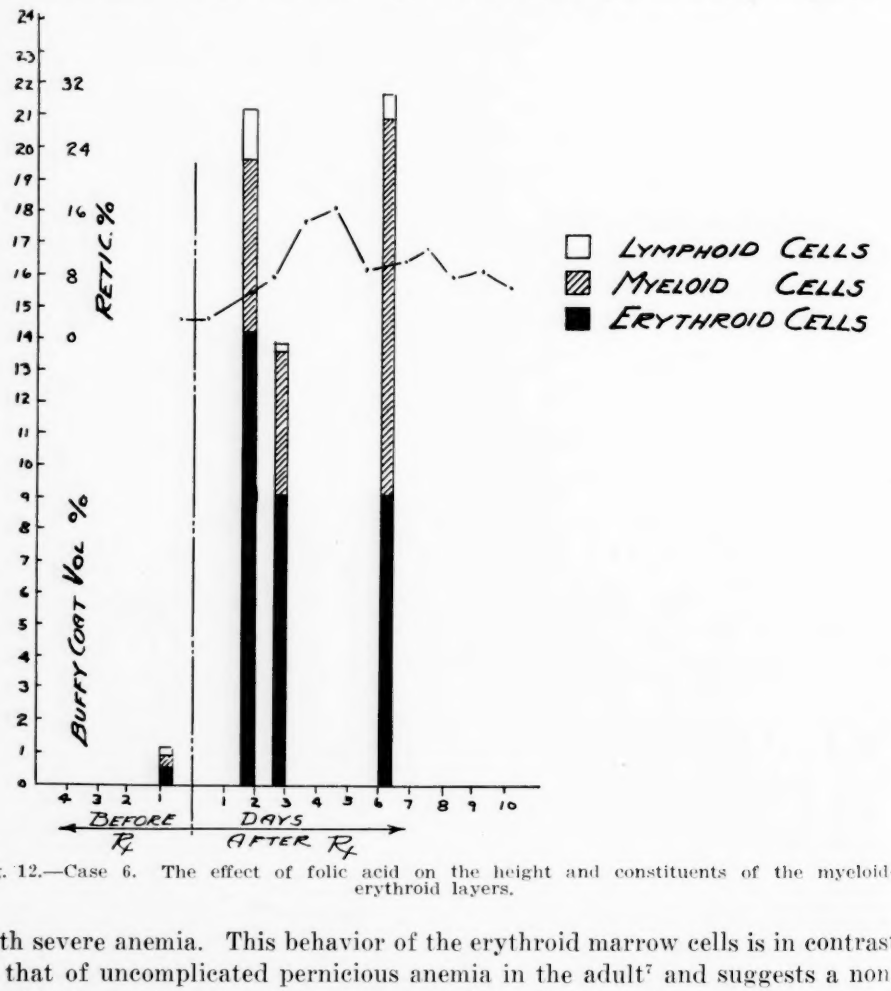

Fig. 12.—Case 6. The effect of folic acid on the height and constituents of the myeloid-erythroid layers.

with severe anemia. This behavior of the erythroid marrow cells is in contrast to that of uncomplicated pernicious anemia in the adult[7] and suggests a non-specific depression of erythropoiesis probably related to the presence of active infection superimposed on the deficiency of hemopoietic principle.

When the erythroid cell volume alone was used as a measure of the response to therapy, a clear-cut increase was demonstrable in twelve of the fifteen cases (Fig. 11), a decrease in two (Cases 4 and 10), and no appreciable

change in one (Case 13). This trend indicates that, for the purpose of a comparative study at least, the method used by us is valid. Using this measure it became evident that in two of the four subjects in whom a drop in the total myeloid-erythroid layer occurred after therapy the decline was not due to a decrease in erythroblasts but to a decrease of other marrow cells (granulocytes and lymphocytes). In one of these subjects (Case 7) the erythroid cells actually increased significantly in spite of the total decrease in myeloid-erythroid volume.

In some instances the rise in the volume of erythroid cells was tremendous, amounting to a thirty fold increase in twenty-four hours in one patient (Fig. 12). Of the two instances in which a drop occurred, one was in a patient (Case 10) whose blood showed reticulocytosis of 8.2 per cent before treatment and who may have been at the beginning of a spontaneous remission. In both patients the initial marrow had shown considerable erythroid hyperplasia before treatment.

There was no apparent proportionality in our material between the extent of the increase and the maximal erythroid hyperplasia achieved on the one hand and the reticulocyte response and hemoglobin response on the other. This lack of correlation may be ascribed to a number of factors operating in our patients; namely, the effects of transfusions, the presence of infection and the effectiveness of the treatment directed against infection, the age differences, the factor of prematurity in one instance (Case 7), and the possible effects of coexisting deficiencies.

SUMMARY AND CONCLUSIONS

Serial studies of the bone marrow in fifteen patients with infantile megaloblastic anemia responding specifically to folic acid therapy as shown by reticulocytosis and rise in hemoglobin and red cell counts demonstrated a spectrum of gradual and progressive aberrations in the appearance of the erythroblasts. The patterns ranged from predominantly normoblastic to frankly megaloblastic cells with many intermediate types which could not be classified in either category. The classic megaloblastic dysplasia is interpreted as only an extreme deviation from normal structure; earlier, less striking changes are significant as shown by the therapeutic test. By the use of more liberal criteria regarding the classification of erythroblasts and by the recognition of constant and specific changes in the granulocytes of the marrow, the disturbance resulting from a marrow deficiency in hemopoietic principle can be detected before the classic megaloblast stage has developed. The lack of hematopoietic principle leads to a disturbance in mitosis, and specific therapy results in a prompt return to normal mitotic activity.

Within twenty-four hours after the intramuscular injection of folic acid clear-cut morphologic changes take place in the marrow of these patients and a response in the peripheral blood can be predicted. Folic acid produces a prompt return to the normoblastic type of erythropoiesis. The immediate transformation of all but the most nearly mature megaloblasts suggests that the megaloblastic dysplasia of the individual cells is reversible so long as the

cell remains capable of mitosis. In the course of the transformation the pathologic cells pass again through an intermediate stage.

In megaloblastic anemia of infancy the total myeloid-erythroid volume of aspirated marrow samples shows no correlation with the degree of anemia and is not a reliable index of the response to therapy, but the calculated volume of erythroid cells shows a direct proportionality to the severity of the anemia and a significant rise in response to therapy.

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FOLIC ACID THERAPY IN NONTROPICAL SPRUE: RESULTS OF TREATMENT IN SEVEN CASES

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DURING the latter part of 1945 Spies and associates[1] reported that synthetic folic acid exerted a favorable influence on the course of macrocytic anemias. Included in their study were patients with tropical sprue in whom the favorable response was not only hematologic but also general. The symptoms referable to the alimentary tract in these patients also tended to subside when folic acid was employed. Subsequent reports include those of Darby and of Spies and their associates[1-4] and occasional instances of sprue in papers of other workers who were chiefly concerned with pernicious anemia.

Davidson, Girdwood, and Innes[5] recently reported results of short periods of treatment with folic acid in ten patients with tropical sprue, idiopathic steatorrhea, and celiac disease. They were primarily interested in the hematologic response which they found disappointing. The clinical response was excellent in the patients with tropical sprue and idiopathic steatorrhea, but improvement was lacking in the patients with celiac disease. Studies of fat balance were made before and after treatment in seven patients (including one with celiac disease). In only one patient was there improvement in absorption of fat.

Nontropical sprue is a condition characterized by diarrhea, steatorrhea, and other gastrointestinal symptoms, macrocytic anemia, glossitis, nutritional manifestations including loss of weight, weakness, hypocalcemia, osteoporosis, tetany, hypoproteinemia, edema, and other changes. These manifestations may vary considerably from patient to patient. The disease, furthermore, is characterized by a chronic progressive course and often prolonged remissions. Treatment in the past has been based chiefly on the use of a low fat diet and administration of liver extract, calcium, and the various vitamins. In general, the results of treatment have been disappointing. In most cases remission occurred, but the tendency to spontaneous remission has rendered the interpretation of the value of therapeutic agents most difficult.

Stimulated by earlier reports of Spies and co-workers[6] our group has treated seven patients who had nontropical sprue with synthetic folic acid.* These form the basis for this report. The criteria of nontropical sprue were fulfilled in the opinion of the several consultants who saw the patients. None of the patients had lived in a tropical country.

PLAN OF STUDY

Control data were obtained before institution of treatment. The history included notes about the frequency, duration, and severity of the exacerbations

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and about the severity of the disease between exacerbations, including frequency and character of stools. Hematologic studies included sternal aspiration and examination, determination of the concentration of hemoglobin in grams, erythrocyte, leucocyte, and reticulocyte counts, and measurement of the erythrocytes with an erythrocytometer. The concentrations of calcium, phosphorus, total protein, albumin, and globulin in the serum, as well as values for phosphatase and prothrombin times, were determined. Roentgenograms of the skull, hands, and teeth for osteoporosis and of the small bowel to exclude ileitis and to check for the so-called deficiency pattern were secured. Roentgenologic examination of the small bowel was not repeated because changes during periods of improvement had not been observed in past experience. In four subjects the fecal solids, fat, and nitrogen were determined each day during the three-day test period while the patient was receiving a standard test diet of 101.6 Gm. of fat, 117.5 Gm. of protein, 269.6 Gm. of carbohydrate, and 2,463 calories. On completion of the preliminary survey a diet was used containing 50 to 100 Gm. of fat and 120 Gm. or more of protein and carbohydrates to raise the caloric intake to 3,000 to 3,500. Vitamins, including sufficient vitamin K to control hypoprothrombinemia, were given. The dosage of folic acid usually was 50 mg. daily by the intramuscular route. One patient received 180 mg. daily intramuscularly for two weeks while another received only 15 mg. orally from the start. Hematologic and chemical determinations on the blood were made each week; the reticulocyte count was taken every other day.

Five of the seven patients were kept under observation at the clinic for four weeks or longer. On dismissal from the clinic the patients continued to take folic acid intramuscularly or orally, the usual vitamins, and, in most cases, calcium lactate in doses of 1 dram (3.9 Gm.) three times daily. Some also subsequently were given liver extract. All were urged to use a high carbohydrate, high protein, low fat diet. The dosage of folic acid is indicated in the individual case reports and in Table I.

Some of the patients subsequently returned for observation and hematologic and chemical studies on the blood. In two subjects the losses of fecal solids, fat, and nitrogen were determined after eight and twelve months of treatment with folic acid. In two others the fecal components could not be determined because of refusal of the patient or because of his condition.

Each subject on his return home was requested to keep a diary in which was recorded the number of stools passed daily as well as other manifestations of the disease.

CASE 1.—A 51-year-old man complained of diarrhea of forty months' duration. The stools were steatorrheal in type and averaged about three daily except during exacerbations, when they numbered as many as ten and fifteen daily. He had lost much strength and 25 pounds (11.3 kilograms). No tetany had been experienced.

Examination revealed undernutrition, for the patient weighed only 110 pounds (50 kilograms), and pigmentation of the skin. Free hydrochloric acid (20 units, by Töpfer's method) was found on gastric analysis. Roentgenologic studies revealed a so-called deficiency pattern in the small intestine. When the standard test diet of 101.6 Gm. of fat and 117.5 Gm. of protein was used, the average daily loss of fat and nitrogen was, respectively, 41 and 5.8 Grams. Stools averaged seven per day during the test period (Table II). An oral glucose

TABLE I. HEMATOLOGIC DATA IN SEVEN CASES

CASE	DURATION OF TREATMENT	FOLIC ACID (MG.)	HEMOGLOBIN (GM./100 C.C.)	ERYTHROCYTES (MIL./C.MM.)	MORPHOLOGY OF ERYTHROCYTES	ERYTHROCYTOMETER (μ)	RETICULOCYTES (% ERYTHROCYTES)	STERNAL MARROW
1	Before		12.3	4.30	Macrocytosis		1	Normoblastic
	1 mo.	50	11.6	3.37	Macrocytosis	8.1-8.3	3.3	
	3 mo.	50	11.2	4.00	Macrocytosis	8.0-8.4		Normoblastic
	9 mo.	50	9.6	3.58	Macrocytosis	8.0		Normoblastic
2	Before		7.6	3.61	Normocytic hypochromic erythrocytes	7.4	1.7	Hyperplastic normoblastic
	1 mo.	50	8.25	3.61		7.6	3.0	
	9 mo.	15	6.4	4.01	Normocytic hypochromic erythrocytes	7.4		
3	Before		12.7	4.30	Macrocytosis	7.8-8.0	1.1-2.2	
	6 wk.	15-180	13.8	4.12	Macrocytosis	7.9-8.1	2.4	Normoblastic
4	Before		10.8	3.09	Macrocytosis		0.4-1.6	Megaloblastic
	6 wk.	50	12.0	3.86	Macrocytosis	8.5-7.8	4.9	Normoblastic
5	Before		11.9	3.03	Macrocytosis	8.3		Macronormoblastic
	2 mo.	50	10.8	3.68	Macrocytosis	8.2	3.0	
	5 mo.	50	10.8	4.08	Macrocytosis	8.2		
	7 mo.	30	9.4	3.30	Macrocytosis	8.2	1.9	
6	Before		10.5	4.25	Macrocytosis	8.6		Normoblastic
	2 mo.	50	10.5	4.29	Macrocytosis	8.3		
7	Before		11.0	5.26	Macrocytosis			Normoblastic
	12 mo.	15	9.8	4.60	Borderline macrocytosis		1.8	

TABLE II. FECAL SOLIDS, TOTAL LIPOIDS, AND TOTAL NITROGEN IN TWO CASES OF SPRUE BEFORE AND DURING THERAPY WITH FOLIC ACID

CASE	RELATION TO FOLIC ACID THERAPY		STOOLS: DAILY AVERAGE NUMBER DURING STUDY	AVERAGE DAILY VALUES*			
	BEFORE	DURING		SOLIDS (GM.)	TOTAL LIPOIDS (GM.)	TOTAL LIPOIDS (% SOLIDS)	TOTAL NITROGEN (GM.)
1	April, 1946		7†	141.7	41.0	28.9	5.8
		Jan., 1947	2	74.2	36.2	48.7	3.8
2	April, 1946		1	50.6	29.6	58.5	2.5
		Jan., 1947	1	81.2	43.9	54.1	2.6
Normal persons	Average			27.6	4.1	14.5	1.7
	Range			13.6-39.1	1.8-6.7	9.3-19.6	0.8-2.5

*A standard test diet containing 101.6 Gm. of fat, 117.5 Gm. of protein, 269.6 Gm. of carbohydrate, and 2,463 calories. Marker technique employed.

†Study conducted during exacerbation of disease.

tolerance test presented a flat curve. The value for serum protein was 6.1 Gm. per 100 c.c. and the albumin-globulin ratio was 1.4:1. Serum calcium measured 8.8 mg. per 100 c.c.; there were 12.3 Gm. of hemoglobin per 100 c.c. of blood, and erythrocytes numbered 4,300,000 per cubic millimeter of blood, of which 1 per cent was reticulocytes. General macrocytosis was present. The sternal bone marrow was normoblastic. Folic acid was given intramuscularly in 50 mg. doses each day. During thirty days of observation the patient did not gain weight, strength, or sense of well-being in spite of a daily intake of 3,000 to 3,500 calories. Frequency of stools did not change; the number varied from one to five daily. At the end of this period the value for hemoglobin was 11.6 Gm. per 100 c.c., and erythrocytes numbered 3,370,000 per cubic millimeter. The highest reticulocyte count was 3.3 per cent encountered on the sixth day of therapy. Throughout this period the erythrocytes measured 8.1 to 8.3 μ in diameter. Serum proteins then measured 6.8 Gm. per 100 c.c., the albumin-globulin ratio was 1.09:1, and the value for serum calcium was 9.3 mg. per 100 cubic centimeters.

After dismissal the patient continued the nutritious diet and daily intramuscular injections of 50 mg. of folic acid. In spite of this treatment he returned in one month because of an acute exacerbation of the disease characterized by abdominal pain, distention, diarrhea, vomiting, abdominal tenderness, and loss of 10 pounds (4.5 kilograms). On examination he was acutely ill and gave evidence of dehydration, acidosis, and azotemia. With correction of these conditions the acute symptoms subsided. He remained under observation another month, during which time administration of the folic acid was continued. He gained a few pounds but mild diarrhea persisted. At the end of this period the concentration of hemoglobin was 11.2 Gm., erythrocytes numbered 4,000,000, macrocytosis persisted with erythrocytometer readings of 8 and 8.4 μ, and the value for serum proteins was 6.1 Gm. per 100 cubic centimeters. On dismissal he was instructed to take 50 mg. of folic acid daily by mouth. This treatment was continued for six more months, during which time the patient did not improve; mild diarrhea with exacerbations periodically continued, and gain in weight did not occur.

The patient then returned for further observation. At this time the value for hemoglobin was 9.6 Gm. and erythrocytes numbered 3,580,000. The general macrocytosis persisted. The erythrocytometer reading as 8.0 μ, the concentration of serum protein was 5.5 Gm. per 100 c.c., the albumin-globulin ratio was 1.1:1, and the value for serum calcium was 8.2 mg. per 100 cubic centimeters. Average daily loss of fecal solids, fat, and nitrogen again was determined (Table II); stools numbered two daily during the test period. Comparison of these data with similar data obtained before treatment disclosed that loss of fat was essentially the same but that nitrogen loss was less during the period after treatment. However, the improvement, in our opinion, is due not to the folic acid but to the state of the disease at the time the data were obtained. More fat was lost during the first period of study and during an exacerbation of the disease than during the second period of study which was made at a time when an exacerbation was not present. The differences are small and probably not significant.

Comment.—The course of the disease has not been changed by treatment with synthetic folic acid, diet, and vitamins. In fact the course has been chronic and gradually downhill in spite of treatment for nine months. Severe exacerbations have occurred; weight has not been gained. The degree of anemia and of hypocalcemia has fluctuated slightly but has remained essentially the same at the last examination as at the first one. Hypoproteinemia has appeared.

CASE 2.—The patient, a woman first seen when she was 29 years of age, had had a lifelong tendency to loose stools but this had been worse during the four years preceding admission. Weight had declined from 130 to 103 pounds (59.0 to 46.7 kilograms), slight edema had occurred, and tetany had been noted for one year. Stools were 64 to 74 per cent fat (dry weight). Gastric analysis revealed 20 units of free acid. Roentgenologic examination of the small bowel revealed a deficiency pattern. The concentration of hemoglobin was 15 Gm. per 100 c.c. of blood; erythrocytes numbered 4,190,000; the value for serum protein

was 5.6 Gm., and for serum calcium, 6.6 mg. per 100 cubic centimeters. Until the next period of observation (period of the present study) when the patient was 33 years old, mild diarrhea had continued; an average of three to four loose stools were passed daily. Slight edema and latent tetany also continued. Weight was 104 pounds (47.2 kilograms). Laboratory studies revealed general osteomalacia of the bones, a flat oral glucose tolerance curve, 7.1 mg. of calcium and 5.9 Gm. of proteins per 100 c.c. of serum, albumin-globulin ratio of 2.2:1, 7.6 Gm. hemoglobin, 3,610,000 erythrocytes, hypochromic normocytic erythrocytes, an erythrocytometer reading of 7.4 μ, and hyperplastic normoblastic sternal marrow. For four weeks a diet of 3,000 to 3,500 calories (fat, 75 Gm.; protein, 150 Gm.) was taken well without significant diarrhea. During this time 50 mg. of folic acid were given intramuscularly daily. A gain of five pounds (2.3 kilograms) occurred. This gain in weight was credited to increased intake of food and to rest. At the end of this period the value for hemoglobin was 8.25 Gm., erythrocytes numbered 3,610,000, the erythrocytometer reading was 7.6 μ, the value for serum protein was 6.6 Gm., the albumin-globulin ratio was 2.18:1 and serum calcium, 7.9 mg. per 100 cubic centimeters. After the institution of folic acid therapy the highest reticulocyte count was 3 per cent on the fifth day of treatment. In general, the patient noted no subjective improvement. She returned home with instructions to continue the highly nutritious diet (3,500 calories with 75 Gm. of fat and 150 Gm. of protein) and to take a multivitamin tablet, iron, and 15 mg. of folic acid daily. At first the folic acid was taken intramuscularly and later, orally.

When the patient returned eight months later, she stated that her weight had increased to 113 pounds (51.3 kilograms) and then had dropped to 96 (43.5 kilograms) because of inability to eat during an exacerbation three months previously. Diarrhea had been troublesome only during the exacerbation, but tetany occurred at intervals and variable edema had been present. Folic acid therapy had been interrupted only for a time during relapse. The weight was 96 pounds. Laboratory studies revealed 6.4 Gm. of hemoglobin per 100 c.c. of blood, 4,010,000 erythrocytes, hypochromic normocytic erythrocytes, an erythrocytometer reading of 7.4 μ, 5.3 Gm. of calcium and 5.3 Gm. of protein per 100 c.c. of serum, and an albumin-globulin ratio of 2.32:1. The patient was placed on the standard test diet both before and after treatment with folic acid, and the values for fecal fat and nitrogen were determined. The severity of the disease apparently was the same during each period of study, the stools numbering one or two daily. After treatment the average daily loss of fats was greater than before treatment but the loss of nitrogen was not significantly different. Therapy with folic acid did not decrease the loss of fat and nitrogen in the feces.

Comment.—Slight gain in weight and improvement in the degree of hypoproteinemia and hypocalcemia occurred, as is so often the case in nontropical sprue, with or without treatment. The temptation to ascribe the improvement to folic acid is disputed by the occurrence of a severe exacerbation while receiving 15 mg. of the drug intramuscularly or orally each day. The course of the disease has not been altered.

Case 3.—A 37-year-old woman had had symptoms of nontropical sprue for three years. Diabetes mellitus had been discovered about the same time. One and one-half years before admission an exacerbation of the sprue syndrome had occurred, during which glossitis was marked and weight had declined from 105 to 78 pounds (47.6 to 35.4 kilograms). Three months later tetany was noted. At the time of admission the patient was not suffering from diarrhea but the stools appeared fatty. Weight was 81 pounds (36.7 kilograms). Laboratory findings were as follows: glycosuria, 1.39 per cent; blood sugar, 185 to 226 mg. per 100 c.c.; osteoporosis of the skull, spinal column, and bones of the hands; and 14 units of free gastric acid. There was roentgenologic evidence of a deficiency pattern in the small intestine. On the standard test diet the average daily losses of fecal solids, fat, and nitrogen were, respectively, 67, 25, and 3 grams. The value for serum calcium was 7.9 mg. and for serum protein, 6.2 Gm. per 100 c.c.; the albumin-globulin ratio was 1.46:1; the concentration of hemoglobin was 12.7 Gm. per 100 c.c.; erythrocytes numbered 4,300,000, of which 1.1 to 2.2

per cent were reticulocytes; general macrocytosis was indicated by erythrocytometer readings of 7.8 to 8.0 microns. A diet containing 2,700 to 3,000 calories, with fat limited to 100 Gm. or less daily, was taken well. The requirements for insulin were met. A gain in weight from 81 to 103 pounds (36.7 to 46.7 kilograms) occurred in seven weeks; about 10 pounds (4.5 kilograms) were gained during the week of observation before folic acid was started. Usually the patient had two or three stools daily but at times constipation was present. After one week's observation folic acid was administered intramuscularly in doses of 15 mg. daily for two weeks, then 30 mg. twice daily for one week, 90 mg. twice daily for two weeks, and finally 90 mg. daily for one week. During this time the gain in weight just mentioned continued. At completion of observation the value for hemoglobin was 13.8 Gm. and erythrocytes numbered 4,120,000; macrocytosis had persisted for the erythrocytometer reading varied from 7.9 to 8.1 microns. The highest reticulocyte count was 2.4 per cent on the seventh day of treatment. Sternal marrow was normoblastic at the time of completion of observation. The value for serum protein was 7.1 Gm., the albumin-globulin ratio was 1.6:1, and the value for serum calcium was 7.9 mg. per 100 cubic centimeters. After return home the patient continued to take folic acid intramuscularly in 15 mg. doses daily and progressed well for three months, when an acute exacerbation of the sprue syndrome developed which led to death.

Comment.—Synthetic folic acid did not alter the course of the disease in this case.

CASE 4.—The patient, a woman, was first seen when she was 24 years of age. Symptoms of nontropical sprue had been present for seven months. Diarrhea, loss of 20 pounds (9.1 kilograms), glossitis, transitory edema, and tetany had occurred. The concentration of hemoglobin was 43 per cent (Dare); erythrocytes numbered 4,060,000. The value for serum protein was 5.45 Gm. and for calcium, 8.7 mg. per 100 cubic centimeters. The value for free gastric acidity was 30 units (Töpfer's method). Stools contained an excess of fat.

When the patient returned seven years later at the age of 31, weakness and tetany were the chief complaints. Diarrhea was present. The blood presented a picture of marked hypochromic macrocytic anemia, the concentration of hemoglobin was 7.0 Gm. per 100 c.c. of blood, and erythrocytes numbered 3,180,000 per cubic millimeter. Mild symptoms continued and at the time of the next visit at the age of 41 the patient complained that diarrhea was troublesome. Weight was then 103 pounds (46.7 kilograms). Free gastric acids measured 36 units. Roentgenologic examination of the small intestine revealed a deficiency pattern. On the standard test diet, the average daily loss of fecal solids, fat, and nitrogen were, respectively, 38, 14, and 2 grams. An oral glucose tolerance test gave a flat curve. The value for serum calcium was 8.0 mg. and for serum protein, 6.2 Gm. per 100 c.c.; the albumin-globulin ratio was 2.06:1. The concentration of hemoglobin was 10.8 Gm. per 100 c.c. and erythrocytes numbered 3,090,000, of which 0.4 to 1.6 per cent were reticulocytes. Macrocytosis was marked. Sternal aspiration revealed megaloblastic marrow. After intramuscular administration of 50 mg. of folic acid daily slight reticulocytosis occurred, the highest reading being 4.9 per cent on the fifth day of treatment. Two weeks later the sternal marrow was normoblastic. After six weeks the value for hemoglobin was 12.0 Gm. per 100 c.c. and erythrocytes numbered 3,860,000. Erythrocytometer readings during the period of treatment varied from 8.5 to 7.8 microns. At the end of the period of observation (six weeks) the value for serum calcium was 9.1 mg. and for serum protein, 7.7 Gm. per 100 c.c.; the albumin-globulin ratio was 1.34:1. During this period of treatment the patient took a 3,500 calorie diet daily (100 Gm. of fat and 120 Gm. of protein). Several mild exacerbations of the diarrhea occurred (seven to ten stools daily); weight increased to 106 pounds (48.1 kilograms), a gain of only 3 pounds (1.4 kilograms).

Following dismissal the patient continued to use a high caloric diet (fat, 50 Gm. and protein, 160 Gm.) and to take 15 mg. of folic acid orally each day. The exacerbations of diarrhea, as well as varying degrees of edema and tetany, have continued to occur, and, on one occasion, a peculiar generalized paralysis occurred. On several occasions during this eight-

month interval hospitalization for treatment became necessary. Liver extract, calcium, vitamins, and intravenously administered amino acids have been used together with folic acid.

Comment.—The condition of this patient remains pitiful in spite of treatment not only with folic acid, but with other agents as well.

CASE 5.—The patient, a 42-year-old woman, had been anemic at varying times for twenty years and had always had a tendency to loose stools. For ten years the stools had at times been more frequent than before and were described as foamy. The chief complaint was fatigue and weakness, and she had lost 8 pounds (3.6 kilograms) in three months. Weight was 128 pounds (58.1 kilograms). Laboratory studies revealed 11.9 Gm. of hemoglobin, 3,030,000 erythrocytes, general macrocytosis with an erythrocytometer reading of 8.3 μ, 9.1 mg. of serum calcium and 6.2 Gm. of serum protein per 100 c.c., and an albumin-globulin ratio of 1.9:1. There was a deficiency pattern in the small intestine on roentgenologic examination. Gastric analysis revealed 52 units of free acid; the oral glucose tolerance curve was flat and the stools contained 31 per cent fat (dry weight) while the patient was taking only 100 Gm. of fat each day. Sternal marrow was macronormoblastic intype. Folic acid in doses of 50 mg. was given orally each day. The highest reticulocyte count was 3 per cent on the eighth day of treatment. After three weeks of treatment the value for hemoglobin was 10.2 Gm. and erythrocytes numbered 3,840,000. After two months of treatment macrocytosis was still present, and the erythrocytometer reading was 8.2 microns. The patient's strength was good but no weight had been gained and episodes of diarrhea continued. Five months after institution of treatment, during an episode of diarrhea, a hemorrhagic state of extensive degree developed and was promptly controlled by administration of vitamin K. During this episode the patient was hospitalized elsewhere and a loss of 10 pounds (4.5 kilograms) in weight occurred. On her return to the clinic the value for hemoglobin was 10.8 Gm., and erythrocytes numbered 4,080,000. Her condition remained fairly stationary for the next month and then the diarrhea became much worse, six to eight stools occurring daily. Weight dropped to 105 pounds (47.6 kilograms). She was again hospitalized and additional measures, including liver extract, were instituted to control symptoms. Folic acid was given intramuscularly instead of orally. Improvement gradually developed. The value for hemoglobin was 9.4 Gm., erythrocytes numbered 3,300,000, the erythrocytometer reading was 8.2 μ, and 1.9 per cent of the erythrocytes were reticulocytes. Stools contained 32.7 per cent fat by dry weight.

Comment.—In addition to absence of response hematologically or clinically to folic acid this patient presented an episode of hypoprothrombinemia with hemorrhage and an acute exacerbation of the intestinal symptoms while faithfully adhering to a regimen which included folic acid.

CASE 6.—A 53-year-old woman had been seen on numerous occasions during the preceding ten years. At the first observation in 1936 cholecystectomy was performed for chronic cholecystitis with cholelithiasis. Exploration of the abdomen was otherwise normal. Moderate gastric retention was present preoperatively and at intervals postoperatively. During convalescence diabetes mellitus became manifest but was readily controlled. Weakness was also a prominent complaint. When the patient was 51 years of age, abdominal cramps, distention, and spells of diarrhea developed. Stools were bulky and light in color. Weight had declined from 99 to 88 pounds (44.9 to 39.9 kilograms). Blood counts which had previously been normal now revealed anemia. There were 11.6 Gm. of hemoglobin per 100 c.c. of blood, 3,340,000 erythrocytes, 6.3 mg. of serum calcium and 6.8 Gm. of serum protein per 100 c.c., and the albumin-globulin ratio was 1.8:1. Stools contained 48.6 per cent fat. The patient received the usual treatment for sprue consisting of a low fat diet, liver extract, calcium lactate, and vitamin supplements. When seen a year later (the time of the present study) symptoms had persisted and weight had declined to 81 pounds (36.7 kilograms). No edema or tetany had been noted and the diabetes had not been a problem. Laboratory study now disclosed 10.5 Gm. of hemoglobin per 100 c.c., 4,250,000 erythrocytes, and general macrocytosis with an erythrocytometer reading of 8.6 microns. The value for serum protein was

6.1 Gm. per 100 c.c.; the albumin-globulin ratio was 1.7:1; the value for serum calcium was 8.2 mg. per 100 cubic centimeters. The fecal solids were 65.3 per cent fat. Sternal marrow appeared normoblastic. In addition to previous therapy, 50 mg. of folic acid were given daily by mouth. Two months later the symptoms and the general status of health and bowel function had not changed. The value for hemoglobin was 10.5 Gm. per 100 c.c., and erythrocytes numbered 4,290,000. The general macrocytosis had persisted; the erythrocytometer reading was 8.3 microns.

Comment.—Folic acid had evidently accomplished little in this period.

CASE 7.—A 17-year-old girl had had episodes of diarrhea since infancy. These occurred about once a month and lasted three to five days; the stools, which numbered ten to twenty daily, were greasy and foamy. Growth was much retarded. She was 4 feet, 10 inches (147.3 cm.) tall and weighed 79 pounds (35.8 kilograms); menses had not been established. Edema had been noted on several occasions. The occurrence of tetany was questionable. The value for hemoglobin was 11 Gm. per 100 c.c., erythrocytes numbered 5,260,000, and macrocytosis was present. The stools were 52.7 per cent fat (dry weight). The value for serum protein was 4.8 Gm. per 100 c.c.; the albumin-globulin ratio was 1.4:1. The concentration of serum calcium was 8.5 mg. per 100 c.c. and the sternal marrow was normoblastic. A regimen consisting of 3,000 calories per day (50 Gm. of fat), liver extract (1 c.c. weekly), and folic acid (15 mg. daily by mouth) was instituted.

The patient returned twelve months later. Monthly exacerbations of diarrhea had continued until two months prior to this visit. Some of the exacerbations were the most severe she had had. She had gained 14 pounds (6.4 kilograms) and had grown 3½ inches (9 cm.). The menstrual periods had not become established although pelvic pain for one to three days each month had occurred. Pigmentation had lessened. The breasts were still undeveloped; some pubic hair was present. The concentration of hemoglobin was 9.8 Gm. per 100 c.c. of blood; erythrocytes numbered 4,600,000 per cubic millimeter, of which 1.8 per cent were reticulocytes. Some hypochromasia and a borderline type of macrocytosis were noted in the blood smear. The value for protein in the serum was 5.9 Gm. per 100 c.c.; the albumin-globulin ratio was 1.99:1. The concentration of serum calcium was 9.5 mg. per 100 cubic centimeters. The stage of bone development corresponded to that of a child 13 years old.

Comment.—Definite improvement occurred in this case and was attributed to improvement in nutrition. It is impossible to determine whether this was due to the improved intake of food or to a natural remission in the disease or to the results of medication. A further period of treatment will be required to determine the principal factor in the improvement, and such a conclusion may not be possible even then.

COMMENT

Most important in the discussion of these cases is a consideration of the general status of the patients' health. Only two patients have shown any significant gain in weight or improvement in strength or sense of well-being (Cases 3 and 7). One patient (Case 3) had had symptoms of nontropical sprue and diabetes mellitus for three years. No diarrhea was present on admission although the stools contained excess fat. Before treatment with folic acid was started she had gained 10 pounds and further gain was accomplished after this treatment was instituted. This gain was considered to be due largely to an increased intake of food and control of the diabetes as the sprue was in remission at the time of admission. At the time of dismissal the patient was taking 15 mg. of folic acid daily by mouth and she progressed well for three months.

Then an exacerbation of the sprue syndrome resulted in death. The other patient (Case 7) showed definite improvement but it is not possible to state the chief factor responsible for this. The patient in Case 1, who gave a three-year history of sprue, continued to take 50 mg. of folic acid daily intramuscularly at home for one month. At the end of this time an exacerbation of the disease caused him to return to our care. He was in a critical condition as evidenced by dehydration, acidosis, and azotemia. In spite of continuation of treatment with folic acid as well as other therapeutic management for six months, his general condition did not improve nor intestinal manifestations decrease. One patient (Case 4) who had had indications of sprue for seventeen years continued to have exacerbations with varying degrees of edema and tetany, and on one occasion a peculiar paralysis, while taking 15 mg. of folic acid daily by mouth. This patient has required frequent hospital admissions during the subsequent eight months. The patient in Case 5, while taking 50 mg. of folic acid daily for six months, has had an episode of hypoprothrombinemia (not uncommon in nontropical sprue) with hemorrhagic manifestations and a subsequent acute exacerbation of the intestinal symptoms of the disease. Two patients (Cases 2 and 6) did not improve during nine and two months, respectively.

In general, the diarrhea and steatorrhea present before treatment with folic acid have continued during treatment, often with severe flare-ups, and there has been no gain in weight, strength, or sense of well-being that could not be attributed to remissions in the disease and improved intake of food. The levels of calcium or protein in the blood and manifestations of tetany or edema, when present, have not been improved permanently in any case, even with improved intake of food or other procedures.

Some anemia was present in all cases at the time of admission, but the value for hemoglobin was 10 Gm. or more in all but one. The lowest erythrocyte count was 3,030,000. No significant changes in blood counts were obtained from treatment with folic acid. Details are given in Table I. General macrocytosis was present in all patients except one, and this patient (Case 2) had the most marked anemia. The macrocytosis persisted after treatment with folic acid in each of the five cases in which re-examination has been done. No change in the erythrocytometer reading was encountered except in Case 4. In this instance the size of the erythrocytes decreased somewhat coincident with a change in the sternal marrow from megaloblastic to normoblastic type. The highest reticulocyte count after commencement of treatment with folic acid was 4.9 per cent on the fifth day of treatment. In general there was little change in the reticulocyte count. However, the degree of anemia on admission was not such that a great response would be expected. In one case megaloblastic bone marrow present before treatment became normoblastic after treatment. In four other cases the sternal marrow was normoblastic before treatment was started. Some of these patients undoubtedly had taken liver extract at varying times before the observations at the clinic. In general, and in spite of this, the hematologic response in this group of cases has been disappointing; the anemia has not improved and the macrocytosis has persisted.

SUMMARY

The following statements seem justified from our observation of this group of seven cases of nontropical sprue. Treatment with folic acid has not resulted in improvement in the sense of well-being or in gain in weight or strength that could not be accounted for by increased intake of food and natural remission of the disease. The frequency or severity of the exacerbations of the intestinal manifestations of the disease has not changed. The hematologic response has been disappointing since anemia and macrocytosis have persisted. All this is in contrast to the favorable results reported in the literature regarding response of tropical sprue to treatment with folic acid. Whether the two conditions are different or whether the condition in our cases had advanced to an irreversible stage and become resistant to treatment with folic acid is impossible to state. These results indicate that, in some cases at least, nontropical sprue does not respond favorably to treatment with folic acid.

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URINARY PHENOLS IN PERNICIOUS ANEMIA

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ALTHOUGH physiologic changes in pernicious anemia are widespread, affecting the hematopoietic, digestive, and nervous systems, the development and course of the disease have not been shown to be closely associated with any specific biochemical disturbance. Therefore, evidence suggesting that the metabolism of the aromatic amino acid, tyrosine, may be altered in pernicious anemia is of considerable interest.

Observations bearing on the metabolic behavior of tyrosine in pernicious anemia indicate that in this disease blood phenol levels are high,[1] the urinary levels of the volatile phenols are lower in relapse than in remission,[2] and the urinary excretion of total phenolic compounds is increased in untreated cases.[3] Jacobson and SubbaRow[4] have reported that l-tyrosine increases the therapeutic activity of their primary antipernicious anemia factor. Since tyrosine is the presumed precursor of melanin pigment, the pigmentation changes frequently observed in pernicious anemia, which include graying of the hair and vitiligo, might also be considered as supportive evidence of the altered metabolism of tyrosine in this disease.

The present study was made to investigate further the metabolism of tyrosine in pernicious anemia by a fractionation of urinary phenolic compounds before and during initial treatment of the disease.

CLINICAL MATERIAL AND METHODS

Seven patients with pernicious anemia were employed in this study. They had not received antianemia therapy for at least six months prior to the present study. All of them had macrocytic anemia with erythrocyte levels below 3,000,000 per cubic millimeter. Histamine refractory achlorhydria was demonstrated in each instance, and all of the patients responded in the expected manner to the administration of antipernicious anemia medication.

Urine specimens were collected for two twenty-four hour periods immediately preceding the institution of therapy and for several twenty-four hour periods at intervals thereafter.

The urinary phenolic compounds were fractionated according to an unpublished method of Shacter and Lewis.[5] By this method, after preliminary hydrolysis, the urine was extracted with ether and the ether-soluble portion extracted first with sodium bicarbonate and then with sodium hydroxide. Phenols were determined by the Medes[6b] modification of the method of Folin and Ciocalteu[6a] on each of the three fractions resulting from this procedure: (1) the ether-insoluble fraction, (2) the ether-soluble-bicarbonate-soluble fraction, and (3) the ether-soluble-bicarbonate-insoluble-hydroxide-soluble fraction. This last fraction will be referred to as the ether-soluble-hydroxide-soluble fraction.

The ether-insoluble fraction contains any tyrosine excreted as such in the urine. The ether-soluble urinary phenolic compounds which are extracted with the weak alkali, sodium

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bicarbonate, are assumed to be, for the most part, aromatic hydroxy acids. Phenol and p-cresol are the only known phenols present in the urine in considerable amounts which are so weakly acidic that they cannot be extracted from another solution except with a strong alkali such as sodium hydroxide.

RESULTS

In Table I the results of the fractionation of urinary phenolic compounds before and after the institution of specific therapy are presented for seven patients with pernicious anemia.

TABLE I. FRACTIONATION OF URINARY PHENOLS IN PERNICIOUS ANEMIA; EFFECT OF THERAPY

		ETHER-SOLUBLE PHENOLS			ETHER-INSOLUBLE PHENOLS	TOTAL PHENOLS
		$NaHCO_3$ FRACT. A	NaOH FRACT. B	RATIO	C	A + B + C
PATIENT	DATE	(MG.)	(MG.)	A:B	(MG.)	(MG.)
G. O.	8/1	318	84	3.8	63	465
	8/2	351	105	3.2	48	504
	8/3*					
	8/4	82	62	1.3	32	176
	8/6	58	154	0.4	40	252
J. O.	5/18	402	25	16.1	48	475
	5/19	362	50	7.2	50	462
	5/20*					
	5/21	70	63	1.1	48	181
	5/24	34	64	0.5	27	125
	5/27	58	76	0.8	32	166
S. T.	6/7	264	32	8.2	24	320
	6/8	283	70	4.0	45	398
	6/9*					
	6/11	91	54	1.9	20	165
	6/14	56	41	1.3	23	120
W. A.	1/7	107	41	2.6	35	183
	1/8	106	52	2.0	31	189
	1/10*					
	1/11	64	60	1.1	40	164
	1/15	40	104	0.4	33	177
S. N.	1/8	240	41	6.0	45	326
	1/9	231	47	5.0	51	329
	1/10*					
	1/12	58	51	1.1	34	143
	1/14	56	48	1.2	39	143
	1/19	64	61	1.0	32	157
B. I.	2/28	245	79	3.3	48	372
	2/29	294	81	3.6	56	431
	3/1*					
	3/4	81	84	1.0	41	206
	3/8	57	101	0.6	48	206

*Treatment instituted on this day. In all patients except B. I. this treatment was 1 c.c. of 15 unit liver extract by intramuscular injection three times weekly. In the case of B. I. the treatment was 40 Gm. of ventriculin daily.

Values expressed as milligrams of tyrosine excreted per twenty-four hours.

In every patient except one (W. A.) the excretion of total phenols was greater in the relapse than in the remission state. This lowering of phenolic excretion levels occurred whether remission was induced by a liver extract or by desiccated stomach (ventriculin). The rapidity with which the excretion of the phenols is decreased should be noted. The decrease occurs within forty-eight hours after the institution of therapy and precedes any change in the corpuscular elements of the blood, including reticulocytosis. These results are

in agreement with our preliminary observations on the excretion of keto acids and hydroxyphenyl compounds by four patients with pernicious anemia which are not included in the present study.[3]

The increased excretion of total phenols in the relapse period is wholly attributable to an increase in the excretion of ether-soluble-bicarbonate-soluble phenols, the fraction containing the hydroxyphenyl acids. The identity of this acid, or acids, has not been established. Although in a previous report,[3] using the method of Penrose and Quastel,[8] we obtained a greater keto acid excretion during the relapse period of pernicious anemia, in this series of patients we were unable to confirm this observation consistently and, in several cases, tests for keto acids in the ether-soluble urine fraction gave negative results.

The ether-soluble-hydroxide-insoluble fraction either remained the same or increased somewhat after therapy. When an increase occurred, it was more apparent in the urine samples collected several days after therapy was first administered. In this connection, Volterra[2] also observed in pernicious anemia an increase in volatile phenols when remission was induced.

An effect of antipernicious anemia therapy to which there is no exception in the cases presented here is the reduction in the ratio of the ether-soluble-bicarbonate-soluble fraction to the ether-soluble-hydroxide-soluble fraction. (This ratio is included in the results in Table I). This ratio reduction occurred even in patient W. A., in whom the total phenol level was not affected.

From Table I it can be seen that the ether-insoluble phenolic fraction remained at a relatively constant level before and during treatment.

The values for total phenols as well as for the various fractions determined during the remission phase of pernicious anemia show good agreement with the values obtained for normal subjects.[7, Table I] It is, therefore, apparent that in

TABLE II. EFFECT OF ASCORBIC ACID ON URINARY PHENOLIC EXCRETION IN UNTREATED PERNICIOUS ANEMIA

PATIENT	DAY	ASCORBIC ACID SUPPLEMENT (MG.)	PLASMA ASCORBIC ACID (MG. %)	ETHER-SOLUBLE PHENOLS		RATIO A:B	ETHER-INSOLUBLE PHENOLS C (MG.)	TOTAL PHENOLS A+B+C (MG.)
				$NaHCO_3$ FRACT. A (MG.)	NaOH FRACT. B (MG.)			
J. O.*	1	0	0.1	147	33	4.4	48	190
	2	0		158	29	5.5	52	195
	3	500		203	32	6.3	47	282
	4	500		402	25	16.1	48	475
	5	500		362	50	7.2	50	462
S. N.*	1	0	0.7	241	57	4.2	46	344
	2	0		246	42	5.1	49	337
	3	500						
	4	500		240	41	6.0	45	326
	5	500		231	47	5.0	51	329
B. I.*	1	0	0.8	301	64	4.7	41	406
	2	0		281	60	4.7	52	393
	3	500		287	78	3.7	48	393
	4	500		245	79	3.3	48	372
	5	500		294	81	3.6	56	431

Values expressed as milligrams of tyrosine excreted per twenty-four hours.

*These patients are also included in Table I. Except for B. I. ascorbic acid supplement was continued during the parenteral administration of liver extract.

pernicious anemia in relapse, the excretion of total phenolic compounds and hence of hydroxyphenyl acids is increased above normal levels.

In one patient with pernicious anemia (J. O.) we observed a low excretion of phenols prior to therapy. This individual had a fasting plasma ascorbic acid value of 0.1 mg. per 100 c.c., determined by the method of Mindlin and Butler.[9] Since ascorbic acid has been shown to have an effect on the oxidation of tyrosine,[10, 11] 500 mg. of ascorbic acid were given daily for a three-day period and the phenol excretion was measured. The results are shown in Table II. On the second day of ascorbic acid administration there was a definite increase in the hydroxyphenyl acid fraction. Ascorbic acid was also given to two patients with untreated pernicious anemia who had normal plasma ascorbic acid values. No changes in either total phenols excreted or in any fraction thereof were observed following the administration of ascorbic acid (Table II).

DISCUSSION

Effective treatment of patients with pernicious anemia with either liver or stomach preparations results in a decrease in excretion of the phenolic fraction containing hydroxyphenyl acids to a level approximating that obtained in normal subjects. This is evidence that substances concerned with erythrocyte maturation participate, directly or indirectly, in the oxidation of phenolic compounds and hence of the amino acid, tyrosine, beyond the aromatic acid stage.

Three of the known vitamins have been shown to affect tyrosine metabolism in various biologic systems: ascorbic acid,[10, 11] pyridoxine as pyridoxal phosphate,[12] and pteroylglutamic acid.[13] Since these vitamins are either absent or present in very small quantities in purified liver extracts,[14] it does not seem possible that the effect of liver extract on phenol excretion can be ascribed to its vitamin content. A more attractive hypothesis is that the antipernicious anemia principle in liver extract activates one or more of these vitamins in some metabolic reaction concerning tyrosine.

In our experiments, administration of ascorbic acid produced no effect on phenol excretion in cases of pernicious anemia where the plasma level of ascorbic acid was normal. In one patient with a low plasma ascorbic acid value and phenolic excretion not increased above normal levels, ascorbic acid administration was associated with an increased phenol excretion. This result is seemingly a contradiction of the well-defined action of ascorbic acid in scorbutic guinea pigs[10] and premature infants[11] in lowering the high excretion levels of hydroxyphenyl acid metabolites. However, indications that close metabolic relationships may exist between the liver principle and ascorbic acid are to be found in clinical reports of "anemia of scurvy"[15] and also in reports of vitamin C having a favorable effect on hematopoiesis in pernicious anemia.[16]

The results of Levine, Gordon, and Marples[11] show that in one of four premature infants, liver extract containing no ascorbic acid resulted in a "transient but definite decrease in hydroxyphenyl compounds." There is a possibility, in view of the experiments reported here, that in this instance the liver principle was the effective agent in reducing phenol excretion. That a deficiency of erythrocyte maturing factors occurs in premature infants is indicated by

reports that such infants often have incompletely developed hematopoietic systems associated with macrocytic anemia.[17, 18]

This study was completed prior to the investigations concerning pyridoxal phosphate and pteroylglutamic acid in relation to tyrosine oxidation; hence the possible effect of these vitamins on phenol excretion in pernicious anemia patients was not determined.

The evidence for aberrant tyrosine metabolism in pernicious anemia leads to the speculation that some phenolic derivative may directly participate in the erythrocyte developmental processes and that the liver principle may affect red cell maturation by virtue of its action in the tyrosine metabolic cycle to produce this derivative. If the formation of some phenolic derivative is essential in the treatment of macrocytic anemias resulting from a maturation defect, this might explain why liver extract is not effective in treating macrocytic anemia occurring in cases of cirrhosis of the liver[19] since tyrosine is not properly metabolized when liver function is impaired.[20]

SUMMARY

The treatment of pernicious anemia with liver or stomach preparations decreases the urinary excretion of total phenols by a reduction in the phenolic fraction containing the hydroxyphenyl acids. There is both a relative and an absolute increase in the fraction containing phenol and p-cresol. The ether-insoluble fraction remains constant.

The values for both total phenols and hydroxyphenyl acids obtained in pernicious anemia during relapse are greater than those obtained with normal subjects.

No change in phenol excretion levels in pernicious anemia in relapse was observed following administration of ascorbic acid to patients having normal plasma ascorbic acid values. In one patient with a low plasma ascorbic acid value, an increase in phenol excretion levels occurred concomitant with ascorbic acid supplementation.

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URINARY PHENOLS IN NORMAL SUBJECTS; EFFECT OF A LIVER EXTRACT

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THIS paper serves as a comparison study for the preceding communication[1] and reports the fractionation of the urinary phenols excreted by normal subjects and the effect thereon of a liver extract.

METHODS AND MATERIAL

The urinary phenol values were determined according to previously described methods[1] on five normal subjects, medical students and dietitians, before and after the injection of liver extract. Throughout this period, to eliminate variations in phenol excretion due to dietary changes, these subjects were kept on a regulated diet similar to that received by the pernicious anemia patients discussed in the preceding report. This diet, on a 2,400 calorie basis, supplied approximately 85 Gm. of protein, 129 Gm. of fat, and 226 Gm. of carbohydrate. Extra calories were provided when needed to maintain weight by increasing the amount of fat and carbohydrate. The diet was supplemented with 200 mg. of ascorbic acid.

The liver extract, given by intramuscular injection, was a purified preparation containing 15 U.S.P. units per cubic centimeter. Thirty units were given in a single dose.

RESULTS

The values obtained by the fractionation of urinary phenolic compounds under these experimental conditions are shown in Table I.

The daily values, before liver extract administration, for total phenols and for the various fractions show considerable variation among the different subjects but are more constant for each individual. The most constant value for each individual is the ratio of the ether-soluble-bicarbonate-soluble fraction to the ether-soluble-hydroxide-soluble fraction.

The results for the two ether-soluble phenol fractions are in fair agreement, both as to quantity and ratio, with those reported by Schmidt[2] who used another method.

At the beginning of the fifth day of the diet, 30 units of liver extract (2 c.c.) were given by intramuscular injection. In the subsequent twenty-four-hour period the excretion of total phenols was reduced in three subjects, remained the same in one subject, and was increased in one subject. The total phenols in most cases tended to increase for the remaining two days of the experimental period.

The excretion of ether-insoluble phenols is not affected by liver extract administration, hence changes in total phenols reflect changes in the ether-

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soluble phenols. When the two fractions of the ether-soluble phenols are considered separately, the effect of liver extract on their urinary excretion level is similar in all subjects. The bicarbonate-soluble fraction usually shows a considerable reduction, while the hydroxide fraction tends to be increased. As a result, the ratio of the bicarbonate-soluble fraction to the hydroxide-soluble fraction is significantly decreased.

The effect of this one dose of liver extract is transient, and the ratio of the ether-soluble phenolic components begins to increase again on the day following the liver extract supplementation.

TABLE I. FRACTIONATION OF URINARY PHENOLS IN NORMAL SUBJECTS; EFFECT OF A LIVER EXTRACT

SUBJECT	DAY ON DIET	ETHER-SOLUBLE PHENOLS		RATIO	ETHER-INSOLUBLE PHENOLS	TOTAL PHENOLS
		$NaHCO_3$ FRACT. A (MG.)	NaOH FRACT. B (MG.)	A:B (MG.)	C (MG.)	A + B + C (MG.)
SE	3	127	70	1.8	40	237
F	4	132	83	1.6	42	257
	5*	59	81	0.7	37	177
	6	70	56	1.3	46	172
	7	117	67	1.6	38	222
KE	2	103	46	2.2	58	207
M	3	105	48	2.2	64	217
	4	106	48	2.2	51	215
	5*	68	53	1.3	56	177
	6	70	48	1.5	51	169
	7	90	51	1.8	49	190
WA	2	69	55	1.3	36	160
F	3	77	62	1.2	38	177
	4	94	65	1.4	31	190
	5*	35	63	0.6	44	142
	6	60	85	0.7	38	183
	7	85	79	1.1	41	205
ST	2	78	59	1.3	51	188
F	3	72	49	1.5	46	167
	4	84	60	1.4	47	191
	5*	47	95	0.5	50	192
	6	59	81	0.7	48	188
	7	70	88	0.8	45	203
BR	3	73	61	1.2	38	172
M	4	82	70	1.2	42	194
	5*	80	98	0.8	44	222
	6	94	91	1.0	40	225
	7	104	82	1.1	38	224

*On this day, 30 units of liver extract (15 units per cubic centimeter) were administered intramuscularly.

Values expressed as milligrams tyrosine excreted per twenty-four hours.

DISCUSSION

The ratio of the ether-soluble phenolic components stands out in this study of phenol excretion under controlled dietary conditions as the most constant value obtained. In other studies on the effects of various substances on phenolic excretion, it might therefore be well to give special consideration to the determination of changes in this ratio.

From the data in the preceding paper[1] it has been shown that the ratio of the ether-soluble phenolic compounds in pernicious anemia patients previous to treatment is much increased over that of the normal subjects. Therapy sharply reduces the ratio, but there is considerable day to day variation. The results of this study show that the administration of liver extract to a normal subject exerts a transient reduction in this ratio, but it is not reduced below the lowest ratio obtained in pernicious anemia in remission.

This transient effect of liver extract on the ratio of the ether-soluble phenolic components excreted by normal subjects may be taken as additional evidence that there is a substance present in liver that acts on the processes of phenolic oxidation either directly or indirectly. There are indications that this substance may be the liver antipernicious anemia principle.

The components of the weakly acidic phenolic fraction (phenol and p-cresol) are commonly regarded as products of enterogenous metabolism, which are absorbed from the gastrointestinal tract and excreted in the urine.[3, 4] In both normal subjects and pernicious anemia patients there were instances where the excretion of this phenolic fraction was increased by liver extract injections. This increase would appear to be the result of an endogenous metabolic process, presumably an effect secondary to the increased oxidation of hydroxyphenyl acids.

SUMMARY

Values for the urinary excretion of various phenolic fractions from normal subjects on a regulated diet have been reported. The most constant value was the ratio for the ether-soluble phenols of the bicarbonate-soluble fraction to the hydroxide-soluble fraction.

When 30 units of liver extract were given, although the amount of total phenols excreted showed no consistent variation, there was a temporary reduction in the ratio of the ether-soluble phenolic components, due both to a decrease in the bicarbonate-soluble fraction containing the hydroxyphenyl acids and an increase in the hydroxide-soluble fraction.

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THE POTENCY OF THE U. S. P. REFERENCE STANDARD FOR VITAMIN D

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ARNOLD[1] has recently published data which indicate that calciferol (vitamin D_2) has a potency of 49.7 ± 2.1 U. S. P. units per microgram in terms of the present U. S. P. reference standard for vitamin D. From this he concludes that the "U. S. P. reference cod liver oil No. 2 has less than its generally accepted potency." If this conclusion is found to be valid, medicinal vitamin D preparations which are assayed by the U. S. P. technique must also be lower in potency than the assays indicate.

Fritz, Hooper, and Moore,[2] in connection with the Association of Official Agricultural Chemists' chick assay of vitamin D_3, have previously called attention to the possibility that the U. S. P. reference standard may be deteriorating. In this connection it may also be pertinent to call attention to the fact that the vitamin A potency of the U. S. P. reference cod-liver oil No. 2 as determined by Callison and Orent-Keiles[3] was lower than its originally assigned value.[4]

Gridgeman[5, 6] has reported the potency of vitamin D_2 to be 36 I.U. per microgram and that of vitamin D_3 to be 46.4 I.U. per microgram. Although Gridgeman compared calciferol to the international standard, while Arnold[1] used the U. S. P. reference standard, Gridgeman's values strike a discordant note, since an overwhelming weight of evidence indicates that vitamin D_2 and D_3 have equal antirachitic activity for the rat. Remp and Marshall,[7] for instance, found vitamins D_2 and D_3 to be equal in potency for the rat. The results of a collaborative study in which nine laboratories participated were reported by Coward[8] and showed vitamins D_2 and D_3 to have equal potency for the rat. More recently, Huber and Barlow[9] have stated emphatically that the two vitamins behave exactly alike as to potency in the rat.

In 1943 Huber and Barlow[9] reported that, on the basis of over 400 satisfactory assays, the antirachitic value of calciferol was well over 40 U. S. P. units per microgram. Some time ago we also observed that samples of calciferol, when compared with the U. S. P. reference standard, consistently had an apparent potency at least 10 to 20 per cent higher than 40 U. S. P. units per microgram. We have therefore repeatedly assayed fresh samples of calciferol from three different sources against the U. S. P. reference cod-liver oil No. 2. The calciferol was dissolved in corn oil and assayed on rats of the Sprague-Dawley strain by the U. S. P. technique.[10] The results are given in Table I.

The initial assays seemed to indicate that the potency of the isolated sample was higher than that of the others. All subsequent assays, however, indicate that the potency of each of the three samples was over 48 and under 52 units per microgram.

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TABLE I. POTENCIES OF CALCIFEROLS

SOURCE OF CALCIFEROL	M.P. (CORR.) ° C.	$E^{1\%}_{1\text{ CM.}}$ AT 265 Mμ	$[\alpha]^{25}_{D}$ IN ETHANOL	APPARENT POTENCY (U. S. P. UNITS PER μG)
Winthop Chemical Co.	115-117	465	+104.5 (c = .978)	50 ± 2
Glaxo Laboratories, Ltd.	115-117	460	+104 (c = 1.018)	50 ± 2
Isolated*	116-117.5	490	+103.7 (c = .982)	50 ± 2

C, grams per 100 milliliters.
*Isolated from ergosterol activated by the Whittier Process.[11]

DISCUSSION AND CONCLUSIONS

The antirachitic potency of the U. S. P. reference cod-liver oil No. 2 was originally determined by assay against the international standard for vitamin D.[4] The U. S. P. unit of vitamin D was, in fact, defined as "equal, in antirachitic potency for the rat, to one International Unit of Vitamin D as defined and adopted by the Conference of Vitamin Standards of the Permanent Commission on Biological Standardization of the League of Nations in June of 1931."[12] The antirachitic potency of pure calciferol, therefore, should be the same whether expressed in international units or in U. S. P units. This appears not to be the case, at least when the U. S. P. standard currently being distributed is used.

The potency of calciferol is recorded in the report of the Second Conference on Vitamin Standardization of the League of Nations[13] as 40 I.U. per microgram. This value was confirmed in a comprehensive study by Anderson, Bacharach, and Smith[14] and by the collaborative study reported by Coward.[8]

Older values obtained by comparative assays against an earlier U. S. P. reference oil[7, 15] indicate that the potency of calciferol at that time was also 40 U. S. P. units per microgram in terms of the then current U. S. P. standard.

The discrepancy between the more recent and the older values for the potency of calciferol might be explained by the assumption that the samples of calciferol more recently available were of a higher degree of purity than the samples with which the older work was done. When the constants of the calciferol used in the aforementioned collaborative study,[8] of the calciferols studied by Anderson, Bacharach, and Smith,[14] and of the "pure calciferol" for which specifications were given in the report of the Second International Vitamin Conference[13] are compared with the constants of the samples of calciferol used by Huber and Barlow,[9] Arnold,[1] and ourselves, it appears that the earlier samples of calciferol were equally as pure as those used more recently.

The only reasonable explanation for the findings of Huber and Barlow,[9] Arnold,[1] and ourselves, therefore, appears to be that the U. S. P. reference standard for vitamin D is below its stated potency. These results and the findings of Callison and Orent-Keiles[3] with relation to the vitamin A potency of the standard strengthen the hypothesis of Fritz and co-workers[2] that the reference standard is deteriorating. It may therefore be advisable to re-evaluate the potency of the U. S. P. reference standard for vitamin D and to re-examine

recent data obtained by comparative assays with this standard. The substitution of a primary standard for the cod-liver oil standard should be considered. We concur in the recommendations of Arnold[1] and of Waddell and Kennedy[16] that pure, crystalline vitamin D_3 be adopted as the reference standard for vitamin D preparations.

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EFFECT OF TYROSINE, TRYPTOPHANE, AND THIOURACIL ON MELANURIA

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THERE has been much speculation on the origin of melanin and melanogen, pigments widely distributed in nature and found in pigmented tumors. Eppinger[1] concluded from feeding experiments with tryptophane and tyrosine in a case of melanosarcoma that tryptophane was the precursor of melanogen. Raper[2] demonstrated that tyrosine was converted to melanin by tyrosinase. Paschkis and co-workers[3] found that thiouracil inhibited the in vitro formation of melanin by tyrosinase.

The purpose of the present investigation was to repeat the experiments of Eppinger and to determine the effect of thiouracil upon the melanin metabolism of a 47-year-old white woman with a melanosarcoma presumably arising from the adrenal gland and metastasizing throughout the body, as revealed by post-mortem examination. In the course of these studies, an improved method for the quantitative determination of urinary melanogen was developed.

METHODS

The urinary melanogen was determined as follows (modification of Thormählen reaction[4]):

> To 5 c.c. of urine, 5 c.c. of a 33⅓ per cent (w/v) solution of sodium nitroprusside were added, followed by 5 c.c. of a 10 per cent (w/v) sodium hydroxide solution, and then by 5 c.c. of a 33⅓ per cent solution of acetic acid. The solution was made up to 500 c.c. with water. Five cubic centimeters of a standard indole solution (containing 0.5 mg. per cubic centimeter) were treated similarly. An A. C. model Fisher electrophotometer with a 650 A filter (with a wave length range of 600 to 700 mμ) was used for the colorimetric comparisons.

Calibration studies with aqueous indole solutions indicated a high degree of conformity of the Thormählen reaction with Beer's law (Fig. 1). Quantitative recovery of added indole from urine was close to 100 per cent in the range of the patient's urinary "melanogen" concentrations (35 to 50 mg. per cent), as shown in Table I.

TABLE I. URINARY INDOLE RECOVERY EXPERIMENTS

ACTUAL CONCENTRATION (MG. %)	OBSERVED CONCENTRATION (MG. %)	AVERAGE OBSERVED CONCENTRATION (MG. %)	PER CENT RECOVERY
15	16.5 16.6	16.6	110.7
30	33.4 32.5	32.9	109.6
50	49.2 47.5	48.4	96.8
65	64.1 63.0	63.6	97.9

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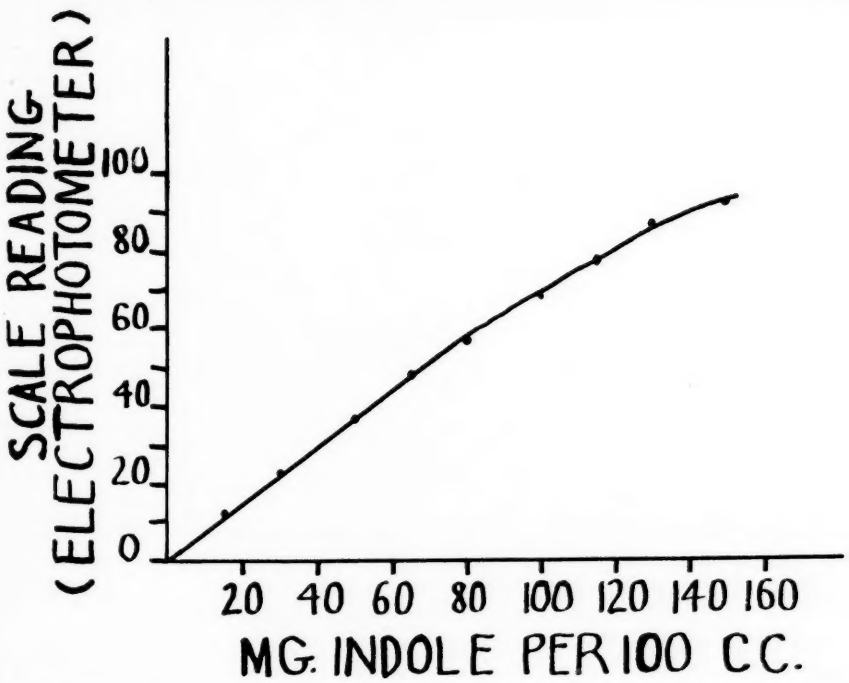

Fig. 1.—Electrophotometric scale reading (scale A) equals 100 × (2 − log per cent transmission).

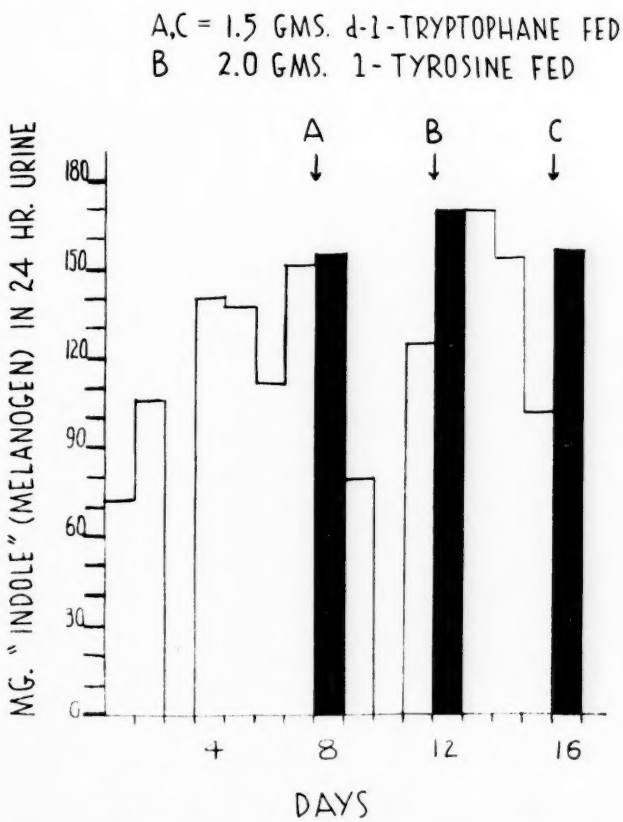

Fig. 2.—The amino acids were fed at *A*, *B*, and *C* at the *beginning* of the respective twenty-four hour periods. No melanogen determinations were performed on the third and tenth days.

Rothman[5] reported that the Thormählen test is negative in indicanuria. I have also observed one subject with indicanuria whose urine yielded a negative nitroprusside reaction. Indican, therefore, is not likely to be confused with melanogen.

EXPERIMENTAL

Our patient was fed 1.5 Gm. of dl-tryptophane, on each of two days, and 2.0 Gm. of l-tyrosine, on another day, as aqueous suspensions flavored with lemon juice.

The results are presented in Fig. 2.

In control studies of twenty-four hour urine specimens collected from ten hospital patients without melanomas and on a regular hospital diet, the Thormählen reaction was negative. In addition, random urines from about 100 patients with diverse diseases yielded a negative Thormählen reaction. Urines collected during the twenty-four hours following the feeding of 1.5 Gm. of dl-tryptophane to each of five normal men and 2.0 Gm. of l-tyrosine to each of five others failed in all cases to give the Thormählen reaction.

After the tyrosine and tryptophane feeding experiments were completed, 0.2 Gm. of thiouracil was administered to our patient three times a day. On the sixth day of drug administration the urine, which had previously been brownish-black, began to lighten in color, and by the eighth day the color approximated that of normal urine. Daily observations were made on each sample for one week or more. These normally colored urines did not darken through the week or more of observation after voiding, even though they were exposed to light (conditions under which melanogen is converted to melanin). The drug was discontinued on the tenth day. Three days thereafter the urine again became black. The patient died at this point, and the experiment could not be repeated.

Thiouracil did not affect the urinary color directly. When 0.2 Gm. of it (several hundredfold excess) was added to 10 c.c. of black urine and incubated at 37° C. for twenty-seven hours, no change in color was observed.

DISCUSSION

Investigators studying patients with melanosarcoma and melanuria have been limited by the fact that such patients do not live very long after the melanuria is detected; in this respect our patient was no exception.

In our patient both tyrosine and tryptophane increased the melanogen excretion slightly above the mean daily output of 122.4 ± 30.0 mg., expressed as "indole," in the control period. After tryptophane was fed 154.9 and 156.4 mg. were excreted; after tyrosine, 169.4 milligrams. These results do not confirm the difference in the behavior of the two amino acids found by Eppinger. It should be noted that his patient was vomiting when she was fed tyrosine, which may account for his failure to observe any increase in the urinary melanogen excretion afterward.

A possible mechanism of thiouracil action in our patient is the inhibition of tyrosinase activity in the tumor cells.

Lynn and De Marie,[6] studying the melanophores of tadpoles, and Juhn,[7] the feathers of brown Leghorn capons, observed decreased pigmentation after thiouracil, but these reactions apparently did not involve the inhibition of tyrosinase.

After this study had been completed, Sugiura[8] reported that daily ingestion of 15 mg. of thiouracil by mice had no inhibitory effect on the growth of transplanted Harding-Passey mouse melanoma.

SUMMARY

1. The feeding of tyrosine and tryptophane to a patient with melanosarcoma and melanuria resulted in a small, possibly significant, increase in the urinary excretion of melanogen.

2. Thiouracil administration caused the urine to change from black to a normal color.

3. The color reverted to black when the drug was discontinued.

4. A possible mechanism of the thiouracil action is the inhibition of melanin formation in the tumor cells.

I wish to extend my thanks for the advice and encouragement given by Dr. Louis Leiter, Chief of the Medical Division, Montefiore Hospital.

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ALLOXAN DIABETES IN THE MOUSE

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ALTHOUGH the effect of alloxan has been studied on a large number of species (rabbit,[2, 6] dog,[8] rat,[5, 9] monkey,[3] man,[4] frog,[14] and duck[13]), to date no reports have been noted on the production of diabetes in the mouse. Inasmuch as it is desirable to use a small diabetic animal when working with expensive materials, as would be the case with tracer substances, this study on mice was undertaken. We were interested in studying the effect of diphosphopyridine nucleotide (D.P.N.) on the action of alloxan and consequently turned to mice to conserve materials. Diphosphopyridine nucleotide and alloxan have been reported to compete with each other in a yeast apozymase system.[10] Since the alloxan inhibition of this enzyme system can be reversed by adding large quantities of diphosphopyridine nucleotide, it was thought desirable to test the effect of diphosphopyridine nucleotide on the production of diabetes with alloxan.

METHOD

Alloxan monohydrate was injected intravenously into the tail vein of the mouse. The mouse was immobilized in a glass cylinder. The tail protruded through a notch in the rubber stopper used to plug one end. An air hole at the opposite end served for ventilation. The entire mouse was warmed on the heating coil of a 56° C. oven for one to several minutes. The induced vasodilatation in the tail facilitated the intravenous injection. A 26-gauge hypodermic needle was used, and blood was drawn into the syringe before starting the intravenous injection. The alloxan was injected as a 1 per cent solution in distilled water. Blood samples (0.05 c.c.) were removed forty-eight hours later by cutting the tip of the tail (after warming the mouse), and the sugar was determined by the Folin-Malmros micromethod.[7]

The diphosphopyridine nucleotide preparation* used for the injections was only 20 per cent pure and was injected in doses of 750 mg. per kilogram intravenously. A separate vein was used for the alloxan which was injected two to five minutes following the diphosphopyridine nucleotide injection.

DATA

Table I shows the effect of injecting various doses of alloxan intravenously in the mouse. The animals were allowed food and water, ad libitum, before and during the experiment. When no alloxan was administered the blood sugar levels of seven of eight mice varied between 169 and 180 mg. per 100 c.c.; only one mouse had a blood sugar value slightly greater than 200 mg. per 100 cubic centimeters. The average forty-eight hour blood sugar is tabulated for the various doses of alloxan used. The average forty-eight hour blood sugar values given in Table I also include those of animals which did not show any blood

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sugar response to alloxan. The incidence of diabetes can be judged by the number of mice with forty-eight hour blood sugar values greater than 250 mg. per 100 cubic centimeters. Mice with mild or questionable diabetes are included in the group whose forty-eight hour blood sugars are greater than 200 mg. per 100 cubic centimeters.

TABLE I. EFFECT OF ALLOXAN INJECTION (INTRAVENOUS) ON MICE

NUMBER OF MICE	DOSE OF ALLOXAN (MG./KG.)	AVERAGE 48-HOUR BLOOD SUGAR	% OF MICE WITH 48-HOUR BLOOD SUGAR (> 200 MG./100 C.C.)	% OF MICE WITH 48-HOUR BLOOD SUGAR (> 250 MG./100 C.C.)
8	0	178	12	0
2	30	203	50	0
7	40	358	71	43
4	50	372	100	50
8	75	464	100	88
2	100	600	100	100

At a dose of 40 mg. per kilogram, three of seven mice had forty-eight hour blood sugar values ranging between 504 and 644 mg. per 100 cubic centimeters. Two showed forty-eight hour blood sugar values slightly greater than 200 mg. per 100 c.c., and the remaining two showed forty-eight hour blood sugar values slightly under 200 mg. per 100 cubic centimeters. Clearly, then, only 43 per cent of seven animals developed significant diabetes at an alloxan dose of 40 mg. per kilogram. When the dose of alloxan was increased to 75 mg. per kilogram, seven of eight mice showed blood sugar values, at forty-eight hours, ranging between 436 and 596 mg. per 100 cubic centimeters. Only one of eight mice showed a blood sugar value at forty-eight hours of 240 mg. per 100 cubic centimeters. Thus, alloxan in doses of 75 mg. per kilogram produced diabetes in nearly all of the mice injected.

Two mice were injected intravenously with diphosphopyridine nucleotide in doses of 750 mg. per kilogram. Inasmuch as the diphosphopyridine nucleotide was only 20 per cent pure, this corresponds to a dose of 150 mg. per kilogram of pure diphosphopyridine neucleotide. Alloxan was injected in doses of 75 mg. per kilogram two to five minutes following the diphosphopyridine nucleotide administration, separate veins being used for each intravenous injection. Blood sugars were determined at forty-eight hours. The two animals receiving diphosphopyridine nucleotide plus alloxan had forty-eight hour blood sugars of 518 and 626 mg. per 100 cubic centimeters. Consequently, no effect could be attributed to the diphosphopyridine nucleotide injection.

DISCUSSION

It will be noted that mice respond to the intravenous injection of alloxan. The dose of alloxan required to produce diabetes in mice seems to be somewhat greater than that required for rats. For in the latter animal, the intravenous injection of alloxan in doses of 40 mg. per kilogram produced diabetes in 95 per cent of the animals injected.[12] As has been previously reported,[12] when alloxan is injected intravenously much smaller doses are required to produce a

given level of diabetes than is the case when the alloxan is injected intraperitoneally. This has also been found true in the mouse.

The amounts of diphosphopyridine nucleotide and alloxan used in these mouse experiments are of the same order of magnitude as the concentrations used by Kensler and co-workers[10] in studying a yeast apozymase system. In these latter experiments an apozymase preparation was suspended in buffer pH 6.5 in the presence of magnesium chloride, glucose, and hexose diphosphate. When the alloxan concentration was 2.2×10^{-4} molar and the diphosphopyridine nucleotide concentration was 15 μg per 4 c.c., the enzyme activity was inhibited to the extent of 92 per cent. However, when the alloxan concentration was kept constant and the diphosphopyridine nucleotide concentration was increased to 525 μg per 4 c.c., only 7 per cent inhibition took place. A diphosphopyridine nucleotide concentration of 525 μg per 4 c.c. corresponds to a concentration of 133 mg. per liter, whereas an alloxan concentration of 2.2×10^{-4} molar corresponds to a concentration of 35 mg. per liter. In the mouse experiments the alloxan was injected in doses of 75 mg. per kilogram to insure diabetes. However, the concentration of alloxan that is actually acting on the mouse cannot be accurately determined. At the pH of the body (7.4), 50 per cent of the alloxan present is converted to a nondiabetogenic compound, alloxonic acid, within the first three minutes.[11] The rate of conversion at pH 6.5 (the conditions used in the apozymase experiments) would be expected to be much less, although the precise rate has not been determined. Alloxan is also reduced to dialuric acid by glutathione.[1] However, the rate of inactivation of alloxan by glutathione in the intact mouse or in the isolated apozymase system studied by Kensler and co-workers[10] cannot be accurately determined. Thus, although the conditions in the mouse experiments cannot be accurately compared with those used in the apozymase system, the amounts of alloxan and diphosphopyridine nucleotide used were of the same order of magnitude. The fact that no protection was observed cannot be taken as evidence that a competition between alloxan and diphosphopyridine nucleotide does not take place in the body, inasmuch as the latter compound may not be able to penetrate the cell membrane; although one might suspect that with beta cell degeneration taking place, the permeabilities might also be changed sufficiently to permit such a compound to penetrate. The presence of phosphatase in the blood may also have played a role in destroying the diphosphopyridine nucleotide administered.

SUMMARY

Alloxan, injected intravenously, produced diabetes in the mouse. With a dose of 75 mg. per kilogram seven of eight animals showed a forty-eight hour blood sugar value ranging between 436 and 596 mg. per 100 cubic centimeters. In two experiments using a large dose of diphosphopyridine nucleotide, injected immediately preceding a diabetogenic dose of alloxan, the course of the diabetes was not altered.

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BACTEREMIA AND ACUTE MENINGITIS DUE TO ALCALIGENES FAECALIS

A Case Report

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ALCALIGENES faecalis is generally considered to be saprophytic and nonpathogenic. However, it seems well established that this organism can produce disease. A review of the literature reveals that *A. faecalis* has been associated with a variety of clinical conditions. Anderson[1] cultured it from the blood of two patients who exhibited a syndrome simulating rheumatic polyarthritis. The organism was isolated from the blood of a patient with acute hepatitis and jaundice by McIntyre.[2] An Alcaligenes type of bacillus was cultured from the blood of a patient with a typhoidlike state by Hazen and Mortillaro,[3] and from the blood of three patients with gangrenous appendicitis by Weiss.[4] It was thought to be the cause of death in a patient with protracted sepsis reported on by Goldberg.[5] The organism has been cultured from gallstones[6] and renal calculi.[7] It has been considered the pathogenic agent in certain types of infant summer diarrhea[8] and the cause of a few cases of adult entercolitis.[9]

There are only four cases reported in which *A. faecalis* was responsible for meningitis. Voorhies and Wilen[10] described a case with *A. faecalis* bacteremia and meningitis which apparently responded to sulfadiazine therapy. Spray and Hawk[11] reported a case of meningitis following otitis media. Mason[12] reported a meningitis complicating a meningocele, and Gatewood[13] described a case of meningitis following a craniotomy.

The case reported herein apparently represents the fifth case of meningitis due to *A. faecalis* to be described in medical literature.

CASE REPORT

A. F. S., a 42-year-old merchant seaman, was admitted to the hospital on May 11, 1946, complaining of fever and weakness. He had been well until four days previously when he first noted intermittent periods of chilliness and fever. The following day he began to cough and noted general malaise and aching over the entire body. The symptoms gradually increased in severity and he vomited on two or three occasions. A few hours prior to admission he began perspiring profusely and noted extreme weakness. The remainder of the history was noncontributory.

Physical Examination.—On admission physical examination revealed a well-developed and well-nourished male who appeared prostrated. Temperature was 37.2° C. (99° F.); pulse, 82; and blood pressure, 132/72. There was mild hyperemia of the pharynx and a few scattered crackling râles were heard over the lung fields.

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Hospital Course.—A tentative diagnosis of atypical pneumonia was made upon admission but the chest x-ray was clear. The patient was treated symptomatically but remained restless and complained of headache and some vertigo upon standing. On the third hospital day the headache became more intense, and slight rigidity of the neck was noted for the first time. The temperature which had been normal except during the first few hours in the hospital rose to 38° C. (100.5° F.). Lumbar puncture revealed a bloody fluid under 210 mm. of water pressure. It was not cultured. The patient was not relieved by the spinal puncture but continued to complain of severe headache and vomited frequently. The temperature, remained about 37.7° C. (100° F.).

On the fourth hospital day the spinal fluid was still bloody and under slightly increased pressure. The patient remained conscious and oriented but complained of headache almost continually and vomited frequently. On the fifth hospital day the laboratory reported growth of a motile gram-negative bacillus with slight tendency to bipolar staining. The patient was then started on streptomycin intramuscularly (2.0 Gm. daily divided into equal doses every three hours) and intrathecally (0.1 Gm. daily) and sulfadiazine. The blood sulfadiazine level was maintained about 10.0 mg. per cent. Streptomycin blood levels were not done.

The organism recovered from the spinal fluid on four successive days and from one blood culture was a motile gram-negative rod that did not produce acid or gas on carbohydrate media, produced an alkaline reaction in litmus milk, reduced nitrates to nitrites, and was indol negative. It was identified by our laboratory as *A. faecalis.* Subcultures from the spinal fluid and blood were also sent to the Bureau of Bacteriology of the Maryland State Department of Health and the identification of the organism was confirmed. The organism was not inhibited by 16 μg per milliliter of streptomycin or by 10 mg. per milliliter of sulfadiazine.

Following the institution of therapy with streptomycin and sulfadiazine the temperature rose over the next two days, reaching 39.5° C. (103.1° F.) on the seventh hospital day. The patient's general condition was otherwise unchanged. However, during this time the spinal fluid changed from bloody to cloudy fluid containing about 1,000 cells and cultures became negative for *A. faecalis.* A blood culture taken just before treatment was started was positive for *A. faecalis.* About the fifth day of treatment the temperature, which had been ranging about 39.5° C. (103.1° F.), began to drop by lysis. The headache decreased in intensity and the patient appeared greatly improved. The subsequent clinical course was one of gradual, progressive recovery.

The intrathecal streptomycin was discontinued after nine days but the intramuscular streptomycin was continued for thirteen days and the sulfadiazine for seventeen days.

Since no portal of entry of the infection was obvious, the patient was carefully surveyed prior to discharge from the hospital. Several routine urine analyses were negative and intravenous pyelogram was normal. *A. faecalis* could not be recovered from several stool cultures. Proctoscopic examination showed no abnormalities. Gastrointestinal series showed slight irregularity of the duodenal cap, but no ulcer could be identified. There was slight spasticity of the sigmoid colon on barium enema, but no diverticuli or other abnormalities could be found.

Laboratory Findings.—The most significant laboratory findings are shown in Fig. 1. Other findings were as follows: On admission the hemoglobin was 14.2 Gm.; white blood count, 16,200 per cubic millimeter with 69 per cent neutrophiles (14 stabs), 3 per cent monocytes, 6 per cent eosinophiles, and 22 per cent lymphocytes. Blood Kline and Kahn tests were negative. Urinalysis showed an alkaline reaction and no significant findings. A heterophile antibody titer was 1:14. Agglutination titers were: typhoid "O," 1:20; typhoid "H," 1:80; paratyphoid A, 1:20; paratyphoid B, 1:40; *Brucella abortus,* negative; and proteus "OX19," negative.

Upon discharge the patient's white blood count was 9,200; hemoglobin, 13.2 Gm.; urine, negative; the spinal fluid and blood cultures showed no growth. Specific agglutinins for *A. faecalis* were not present in the blood at any time.

Follow-up Examination.—The patient was readmitted to the hospital on Nov. 2, 1946, for follow-up examination, approximately five months following discharge. At that time he stated that he was feeling well. He complained only of occasional frontal headaches and mild

vertigo. Complete physical examination, including detail neurological survey, was completely negative. Lumbar puncture revealed a clear fluid under normal pressure and with normal dynamics. The cell count was 0 and the globulin was negative and there was no growth on culture.

The patient was again observed on May 20, 1947, about one year after the illness. Complete neurological examination was again essentially normal.

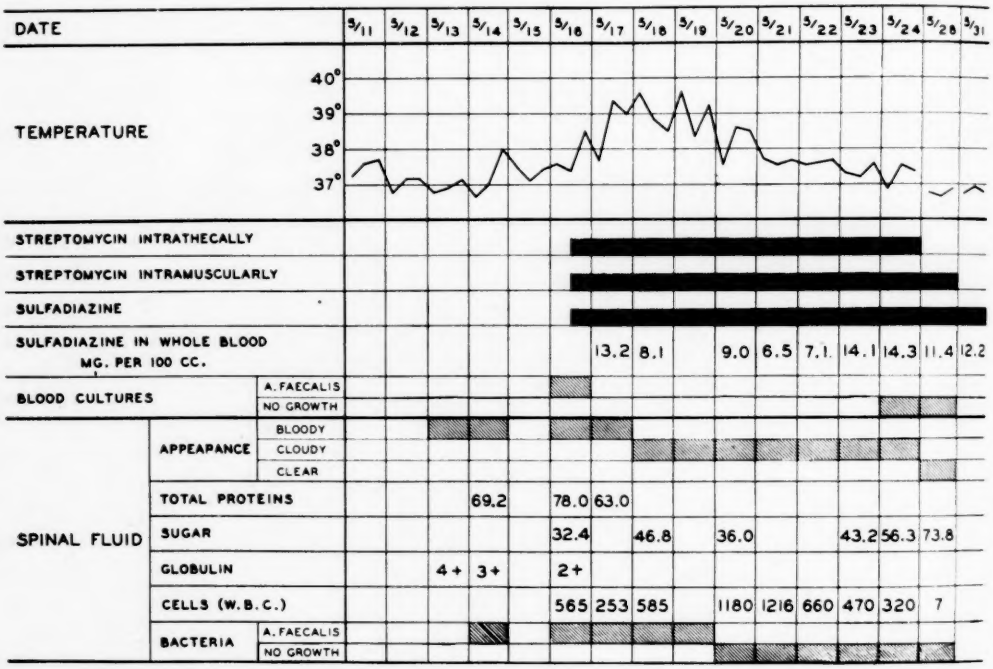

Fig. 1.—Summary of pertinent clinical and laboratory data on a case of *A. faecalis* bacteremia and meningitis.

COMMENT

The isolation of *A. faecalis* in pure culture four times from the spinal fluid and once from the blood seems to establish a case of bacteremia and acute meningitis due to this organism. Though *A. faecalis* is fairly frequently encountered in stool cultures, it is generally not thought capable of producing disease. A few instances to the contrary have been cited herein. This case serves to illustrate that *A. faecalis* can produce disease under certain circumstances. Unfortunately we are unable to attribute this pathogenicity to a lack of resistance on the part of the host or to a proved increased virulence of this particular strain or organism. There was no known event to suggest a decrease in resistance of the host, and animal virulence tests were not done with the cultures of *A. faecalis*.

The part therapy played in the recovery of this patient is also uncertain. The in vitro sensitivity tests on the organism revealed that growth was not inhibited by 16 μg per milliliter of streptomycin or by 10 mg. per milliliter of sulfadizine. Both of these figures indicate considerable resistance on the part

of the organism, and generally one does not expect a favorable response to treatment under such circumstances. The slow clinical response in this case casts considerable doubt relative to the effect of either streptomycin or sulfadiazine. However, there may have been some synergistic activity between the two. One would not have expected recovery without some specific therapeutic effect, but spontaneous recovery cannot be disproved.

Textbooks of bacteriology express doubt relative to the production of specific agglutinins for *A. faecalis* in patients with infections due to this organism. However, Wyatt[14] and Stuart, Thompson, and Krikorian[7] have found agglutinins present in low titer. The case reported by Voorhies and Wilen[10] did not show agglutinins. They were not demonstrable in this case.

SUMMARY

A case of acute bacteremia and meningitis due to *A. faecalis* is described. Despite high in vitro resistance of the organism to both streptomycin and sulfadiazine alone, the patient recovered under treatment with these two agents; synergistic activity may have been present. No preceding infection or port of entry for *A. faecalis* could be demonstrated. Agglutinins for *A. faecalis* could not be demonstrated in the blood after recovery. Follow-up examinations five and twelve months after the onset of illness failed to reveal any abnormalities.

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LABORATORY METHODS

THE THYMOL TEST OF MACLAGAN

Standardization and Adaptation to the Evelyn Photoelectric Colorimeter

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IN THE course of work with the thymol test of Maclagan,[1, 2] started early in 1945, it soon became apparent that the visual estimation of turbidity was not convenient for precise investigation.

Standards prepared according to King and Haslewood,[3] obtained from two different sources and also from our laboratory, were slightly different. Moreover, comparison in the case of lipemic or deeply icteric sera was difficult or impossible.

In an attempt to obviate these disadvantages, a blank prepared with serum and 0.85 per cent sodium chloride (1 to 60 volumes) was introduced for each determination. Although this modification made easier the measurement of the turbidity of sera with a high bilirubin or fat content, it did not eliminate the errors inherent to visual methods of nephelometric analysis.

In order to use the photoelectric colorimeter for the turbidimetric determinations, an easily reproducible and stable calibration was necessary. Serum plus sulfosalicylic acid and barium sulfate were found unsuitable as standards, due to great variation in the results obtained. Finally a copper sulfate solution was adopted for calibration; it is easily prepared and gives in different experiments almost identical readings at 660 μ.

In this study the Evelyn photoelectric colorimeter has been used.

EXPERIMENTAL

Choice of Filter.—

Measurements at 660 μ are very convenient, due to little interference with bilirubin and hemoglobin light absorptions and due to enough sensitivity for turbidity. Shank and Hoagland[4] have emphasized these points.

Calibration.—

Serum With Known Protein Content: The turbidity developed when 3 per cent sulfosalicylic acid is added to diluted serum is the method recommended to check the gelatin standards in the thymol test.[2]

Sera of known protein concentration (Kjeldahl in duplicate) were diluted with 0.85 per cent sodium chloride, so as to obtain solutions corresponding from

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8 to 200 mg. of protein per cent; that is to say, from 0.8 to 20 thymol units. Three volumes of 3 per cent sulfosalicylic acid were added to each tube, and the readings were made in the Evelyn photoelectric colorimeter with No. 660 filter.

The results obtained with different sera and also with the same serum at different moments were very variable, as shown in Fig. 1.

This inconsistency of the turbidimetric measurements of serum protein has also been demonstrated for different albumin preparations by Shank and Hoagland.[4]

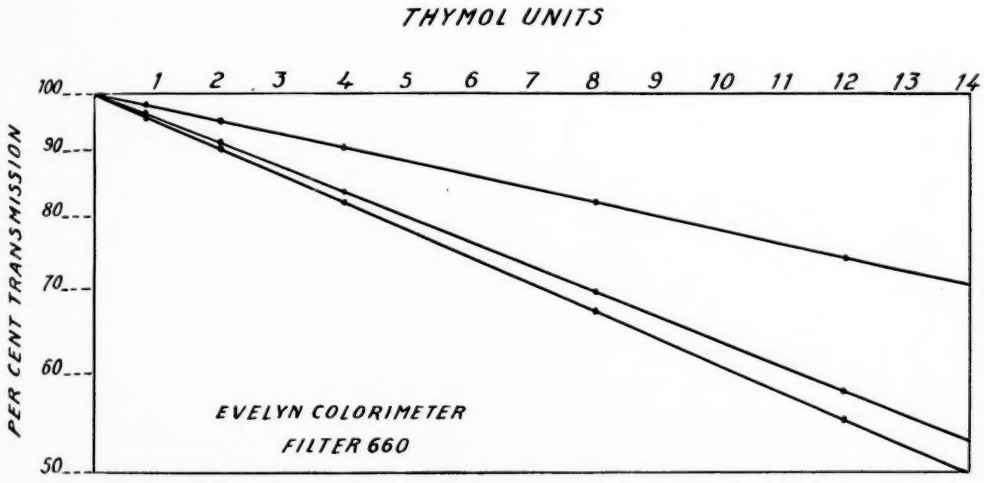

Fig. 1.—Calibration with diluted serum plus sulfosalicylic acid. Only three illustrative examples are reproduced. Values are expressed in thymol units (milligrams of protein divided by ten).

Barium Sulfate: Shank and Hoagland[4] have advocated a barium sulfate standard for the calibration of the spectrophotometer in the thymol test.

When this procedure was first employed very high values were found, but this was due to a typographic error in the original paper. The barium chloride solution is 0.0962 M (2 per cent) instead of 0.0962 N (1 per cent), according to the correction in the reprints.

Calibration with the barium sulfate standard is also variable, even if the same reagents and the same temperature are used, as shown in Fig. 2. This is probably due to different size of the precipitate particles and renders this method inconvenient for photoelectric calibration.

Thymol Purity.—

The chemical purity of the thymol crystals has certain importance in the turbidity values obtained. Using two different brands, discrepancies were found in a great number of sera studied, as shown by the typical examples given in Table I. The reagents were prepared with the same chemicals and precautions, except for the thymol crystals.

Blank.—

Even if the No. 660 filter is used in the photoelectric measurements, it is convenient to run a serum blank to eliminate the influence of natural opalescence,

TABLE I

SERUM	THYMOL CRYSTALS FROM FRITZCHE BROTHERS, INC. (NEW YORK) (UNITS)	THYMOL CRYSTALS FROM MERCK (DARMSTADT) (UNITS)
1	6.6	7.6
2	11.06	14.18
3	1.6	1.9
4	10.44	15.18
5	10.8	12.56
6	3.1	4.3
7	5.1	5.6
8	11.3	14.56

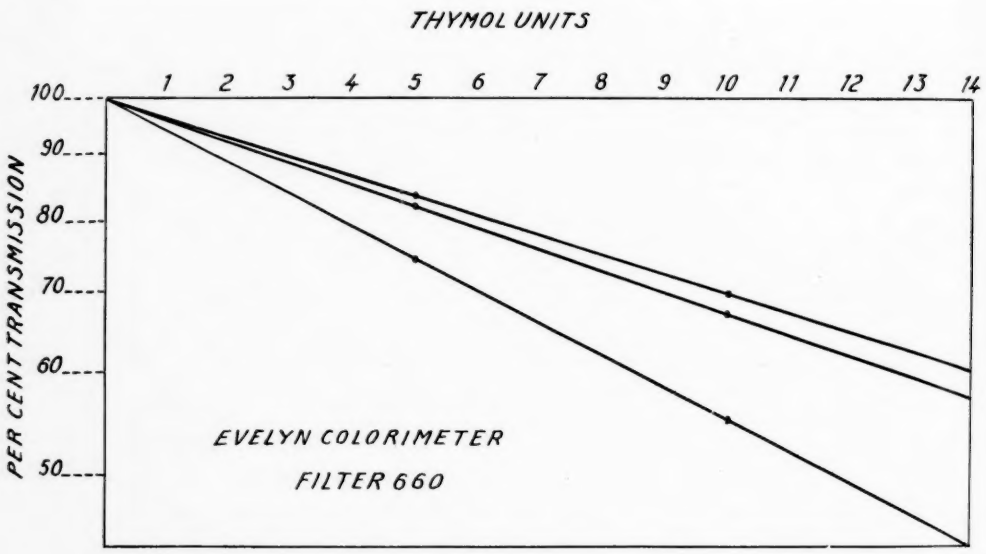

Fig. 2.—Calibration with barium sulfate. Only three illustrative examples are reproduced. Values are expressed according to Shank and Hoagland,[4] and in thymol units.

hemolysis, or high bilirubin content completely. This is shown in the typical examples given in Table II where readings using thymol buffer and serum plus saline as blanks, are compared.

The use of a serum-saline blank depends on having a clear thymol buffer. In our experience it has never become opalescent or turbid on standing, but this can happen under certain conditions apparently related to climate. Watson,[5] for instance, stated: "The thymol buffer solution is not as constant as one might wish; its turbidity varies a little from batch to batch, and from time to time. We find that on standing it becomes visibly more turbid over a period of weeks, and that this cannot be removed by filtration." Schloss[6] also refers to variations of the thymol reagent.

If one has to work with a more or less turbid thymol buffer, it is more convenient to use it as a blank instead of serum-saline.

After many trials, the following calibration has been adopted. It is very easy to perform and, although it is purely empirical, the values obtained are

TABLE II

SERUM	BLANK	
	THYMOL BUFFER (UNITS)	SERUM PLUS SALINE (UNITS)
1	4.3	5.1
2	2.96	1.39
3	2.13	2.5
4	6.2	5.4
5	12.06	14.56
6	3.2	2.89
7	3.5	2.8
8	5.0	3.8

similar to those reported by Maclagan with the visual estimation. A blue solution has been used since the readings for turbidity are taken with a red (No. 660) filter.

METHOD

Calibration.—A 5 per cent aqueous solution of copper sulfate ($CuSO_4.5H_2O$) is prepared and filtered if necessary. To 2 ml. in an Evelyn tube, 8.5 ml. of distilled water are added. From this diluted solution, 3 ml. and 1.5 ml. are placed in a second and third Evelyn tube, respectively, and the volume is taken up in both to 6 ml. with distilled water. The readings are made in the Evelyn colorimeter, using the No. 660 filter, the 6 c.c. aperture, and bright light, with distilled water as a blank. The equivalence of the standards in thymol units and a typical example of calibration are shown in Table III.

TABLE III

2 ML. OF 5 PER CENT $CuSO_4.5H_2O$ + 8.5 ML. OF DISTILLED WATER	DISTILLED WATER	UNITS	G
6 ml.	-	20	54′
3 ml.	3 ml.	10	73
1.5 ml.	4.5 ml.	5	85

The straight line relationship between the optical density of the standards is shown in Fig. 3. For convenience, a table with the correspondence between G values and thymol units is reproduced (Table IV).

The intensity of the blue color is not dependent upon the brand of copper sulfate or the time elapsed between preparation of the solution and colorimetric readings. Salts from three different sources (Baker; Schering, Kahlbaum; and Merck, Darmstadt) gave identical G values soon after preparation or after standing overnight at room temperature.

Procedure.—The thymol buffer is prepared as described by Maclagan,[2] using reagent grade thymol crystals. As the final pH of the solution somewhat varies, it is convenient to adjust it to 7.8. The buffer is stored at a temperature over 20° C., as recommended by Maclagan. On standing over a long period, the thymol solution may show a small amount of precipitate at the bottom of the flask; although it could still be used, it is better to discard it.

In each of two Evelyn tubes, 0.1 ml. of serum is measured; 6 ml. of thymol buffer and 6 ml. of 0.85 per cent sodium chloride are added, respectively. After

TABLE IV. EQUIVALENCE BETWEEN GALVANOMETER READINGS (G) IN THE EVELYN PHOTOELECTRIC COLORIMETER, AND THYMOL UNITS (FILTER NO. 660, 6 C.C. APERTURE, AND BRIGHT LIGHT)

	0	1	2	3		0	1	2	3
99	0.4	0.33	0.23	0.19	73	9.9	9.8	9.7	9.6
98	0.7	0.63	0.56	0.49	72	10.3	10.2	10.1	10.0
97	1.0	0.93	0.86	0.79	71	10.8	10.68	10.56	10.44
96	1.3	1.23	1.16	1.09	70	11.3	11.18	11.06	10.94
95	1.6	1.53	1.46	1.39	69	11.8	11.68	11.56	11.44
94	1.9	1.83	1.76	1.69	68	12.3	12.18	12.06	11.94
93	2.2	2.13	2.06	1.99	67	12.8	12.68	12.56	12.44
92	2.5	2.43	2.36	2.29	66	13.3	13.18	13.06	12.94
91	2.8	2.73	2.66	2.59	65	13.8	13.68	13.56	13.44
90	3.1	3.03	2.96	2.89	64	14.3	14.18	14.06	13.94
89	3.5	3.4	3.3	3.2	63	14.8	14.68	14.56	14.44
88	3.9	3.8	3.7	3.6	62	15.3	15.18	15.06	14.94
87	4.3	4.2	4.1	4.0	61	15.8	15.68	15.56	15.44
86	4.7	4.6	4.5	4.4	60	16.4	16.25	16.1	15.95
85	5.1	5.0	4.9	4.8	59	17.0	16.85	16.7	16.55
84	5.5	5.4	5.3	5.2	58	17.6	17.45	17.3	17.15
83	5.9	5.8	5.7	5.6	57	18.2	18.05	17.9	17.75
82	6.3	6.2	6.1	6.0	56	18.8	18.65	18.5	18.35
81	6.7	6.6	6.5	6.4	55	19.4	19.25	19.1	18.95
80	7.1	7.0	6.9	6.8	54	20.0	19.85	19.7	19.55
79	7.5	7.4	7.3	7.2	53	20.6	20.45	20.3	20.15
78	7.9	7.8	7.7	7.6	52	21.2	21.05	20.9	20.75
77	8.3	8.2	8.1	8.0	51	21.9	21.73	21.56	21.39
76	8.7	8.6	8.5	8.4	50	22.6	22.43	22.26	22.09
75	9.1	9.0	8.9	8.8	49	23.3	23.13	22.96	22.79
74	9.5	9.4	9.3	9.2	48	24.0			

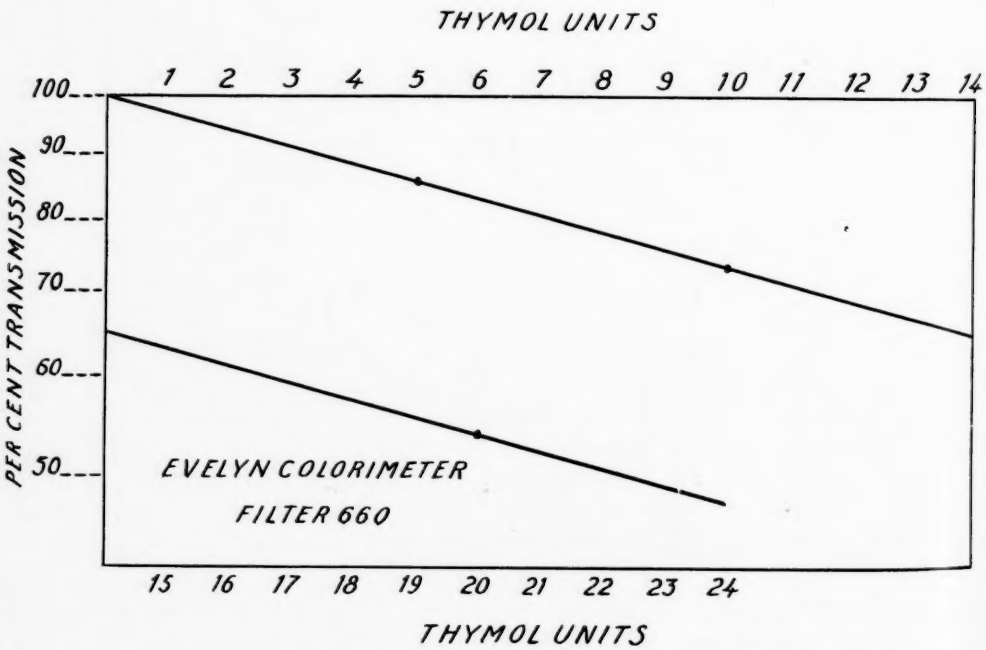

Fig. 3.—Calibration with copper sulfate. Values are expressed in thymol units.

standing for one-half to one hour, readings are taken in the Evelyn colorimeter (No. 660 filter, 6 c.c. aperture, and bright light), using the serum-saline tube as a blank. If the thymol solution is more or less turbid, a buffer blank is substituted for the serum-saline one. The value in thymol units is obtained from Table IV. It is convenient to shake the serum-thymol tube gently before placing it in the colorimeter, for some sedimentation may occur even in this short time.

After completion of the readings, the content of the serum-thymol tube is placed in a small Pyrex tube and left undisturbed overnight to allow observation of the amount of precipitate formed. This is graded from 0 (absent) to 3 plus; no 4 plus value has been used for some of the precipitate remains adherent to the walls of the tube, even with strongly positive sera.

NORMAL AND PATHOLOGIC VALUES

Using the method described, the range found in 100 apparently normal subjects was from 0.4 to 4.0 units.[7, 8] In only five of these cases some precipitate occurred after eighteen to twenty hours; four were recorded as 1 plus, and one as 2 plus.

In 152 of 170 cases of hepatocellular jaundice studied in the acute phase of the disease, the thymol turbidity test was over 4 units (89.4 per cent). Of the total, 111 were examples of infectious (sporadic) hepatitis, and fifty-nine, of cirrhosis with clinical icterus (thirty-four proved by punch biopsy, peritoneoscopy, or post-mortem examination). In 113 of these same cases (66.4 per cent) some degree of flocculation was observed.[9]

The thymol turbidity test was negative in twenty-two of thirty-five cases of obstructive jaundice (62.8 per cent) in which the diagnosis was confirmed by operation, necropsy, or punch biopsy; thirteen cases were due to cancer, nineteen to coledocolithiasis, and three to benign stricture of the common duct. Flocculation was negative in thirty; three of the positive results were 3 plus, and two, 2 plus.[9]

DISCUSSION

The thymol test of Maclagan is a useful complement to the laboratory study of diseases of the liver and bile ducts, as demonstrated by various reports.[2, 4, 8-13]

A drawback of the test is the visual estimation of turbidity advocated by Maclagan.[1, 2] Moreover, the gelatin standards of King and Haslewood[3] are not easy to prepare and vary in opalescence when they are obtained from different sources.

Albumin plus sulfosalicylic acid and barium sulfate have been demonstrated unsuitable for calibration on the colorimeter because they give inconsistent results.

Recently, Kunkel and Hoagland,[14] besides stating that the barium sulfate suspension has certain disadvantages, have advocated Evans blue dye (T-1824) as a standard of reference. Although this calibration is based on the same general principles of the one here presented, the concentration of 3 γ per cubic centimeter (equivalent to 20 units) appears overestimated, for normal sera give values up to 8 units. It corresponds approximately to 10 units, using the copper sulfate solution as reference.

Ley and associates[15] have described an expression of the values for turbidity as cubic centimeters of barium chloride suspension. Besides being purely arbitrary, and based on barium sulfate obtained from 1 per cent barium chloride, it is subjected to the same criticisms as is Shank and Hoagland's calibration. There is no advantage in eliminating the original expression of results in units.

Additional data are needed to appreciate the value of the thymol flocculation. It has been less often positive, both in hepatocellular and obstructive jaundice, than the thymol turbidity. Apparently thymol flocculation is more useful in the follow-up of chronic hepatitis, as recently demonstrated by Neefe.[16]

SUMMARY

A modification of the thymol test of Maclagan is presented. The values for turbidity are obtained in the Evelyn photoelectric colorimeter, using No. 660 filter and a blank of serum plus physiologic saline. A buffer blank is employed when the thymol solution is more or less turbid.

The instrument is calibrated with a copper sulfate solution and the values expressed in units as originally advocated.

This calibration is easily performed, is independent of the salt brand, and is reproducible.

Flocculation is observed after eighteen to twenty hours and is expressed as 0, 1 plus, 2 plus, or 3 plus.

Normal values with this method are exactly those reported by the originator of the test.

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THE COLLOIDAL RED TEST FOR THE STUDY OF HEPATIC DYSFUNCTION

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THE colloidal gold test, as modified by Maclagan,[1] is a valuable help in the laboratory study of hepatic disease.[1-4] Nevertheless, the preparation of the gold sol, used as a reagent in the test, has to be done with meticulous care, following many technical details, which renders it a cumbersome procedure.

Recently, Maizels[5] has suggested the use of a colloidal suspension of scarlet red which is added to three different dilutions of serum. In this test characteristic changes in color are observed in normal and in pathologic conditions. The main drawback of this reaction is the difficulty that arises in reading the results when icteric, hemolyzed, or opalescent sera are used.

After many trials, we[6] have adopted a test in which a modified red sol is used and which is carried out following the general principles of Maclagan's colloidal gold reaction. A buffer with a lower pH is used for the colloidal red suspension is more stable than the gold sol.

According to our present experience, the results of this test are the same as those obtained with Maclagan's colloidal gold reaction, and the red test has the advantages of being more reproducible and much simpler.

COLLOIDAL RED TEST

Reagents.—

1. Stock scarlet red solution: A saturated alcoholic solution of scarlet red (Scharlach R, Sudan IV)† is made by putting some of the powdered dye in a bottle and dissolving it in absolute ethyl alcohol. It is stored in the incubator at 37° C. at least for twenty-four hours before using. The solution must be saturated.

2. Buffer: (pH 7.53) 0.325 Gm. of barbital-sodium (sodium diethylbarbiturate; $(C_2H_5)_2:C:(CONNa.CONH):CO$), 0.639 Gm. of barbital (acid diethylbarbituric; $(C_2H_5)_2:C:(CONH)_2:CO$) and 0.2 Gm. of phenol (carbolic acid; C_6H_5OH) are dissolved in 90 ml. of distilled water with the aid of heat, and after cooling, the volume is taken up to 100 milliliters. The pH is then checked and adjusted if necessary.

3. Scarlet red reagent: 10 ml. of the stock scarlet red solution in a 250 ml. Erlenmeyer flask and 50 ml. of distilled water in a 100 ml. Erlenmeyer flask are warmed to about 55° C. The water is then quickly added to the dye solution. The mixture is boiled first in the water bath and then over gauze till the final volume is about 20 milliliters. The volume is then made up to 800 ml. with distilled water. The reagent is kept in the refrigerator.

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†The "Harleco" dye, prepared by Hartman-Leddon Co., Philadelphia, Pa., has been found suitable for the test.

Procedure.—

In a small Pyrex test tube, 0.05 ml. of serum and 0.5 ml. of buffer are placed. After mixing, 2.5 ml. of scarlet red reagent are added. The results are read, after the tubes have been left undisturbed at room temperature overnight, according to the following:

0 = No change
1 = Faint precipitate, visible only after shaking
4 = Supernatant fluid slightly colored, with heavy precipitate
5 = Clear supernatant fluid with heavy precipitate
(2 and 3 are intermediate grades of flocculation, mainly judged by the color of the supernatant fluid)

The data obtained by using this test, as compared with the cephalin flocculation, the colloidal gold test, the thymol test, the Takata-Ara reaction, and the Gros test, will be the subject of separate communications.[7]

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PRODUCTION OF POTENT BLOOD GROUPING SERA

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THE procurement of potent blood grouping sera is a problem of importance to all those interested in research in blood groups and during World War II was also a source of concern to the military authorities. It was not until 1944 that Witebsky, Klendshoj, and McNeil[7] published a simple method which seemed to meet the demands. This involved the injection of small amounts of a mixture of A and B substances of animal origin into human volunteers of groups A and B. Since that time a number of workers[2-4] have shown the usefulness of the method.

The present paper is designed to report the results of another routine application of the method of Witebsky and co-workers[7] and to comment briefly on the safety and suitability of the procedure. From a group of medical students nine volunteers were selected and each given an intravenous injection of 0.9 c.c. of saline mixed with 0.1 c.c. of a solution of mixed A and B substance* (7 mg. of A substance drived from hog stomach, and 5 mg. of A and B substance derived from horse stomach). No women were chosen because of the (very remote) possibility that the injection might at some later date favor sensitization to A or B factors during pregnancy,[5] but otherwise no selection was exercised, and the first ten men who volunteered were chosen. One of these later became apprehensive and dropped out of the experiment.

In my first use of these A and B materials, fear of an allergic type of reaction following the injection was not entirely absent from my mind at any time.[2] No such reaction has ever occurred, however, and it is interesting to record that Dr. S. B. Hooker, who before injection tested the present group of volunteers for skin sensitivity to the A and B materials as well as to pork and horse and human salivas of groups A and B, found nearly every individual to exhibit some skin reactivity to one or more of the test substances. One of the subjects exhibited a rather marked reaction to pork. Nevertheless, none of the subjects noticed any reaction, either immediate or delayed, following the injection. The danger from the procedure, judged by the total absence of symptoms in a large number of experiments in addition to those reported here, must be considered negligible.

The increases in isoagglutinin titer are shown in Table I. There was no difficulty, even with such a small group, in selecting from titrations of the trial bleedings one satisfactory A donor and one satisfactory B donor, which was all that was desired. From this and previous work the impression has been gained that about one-half of the volunteers respond with increases of titer and

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*Supplied by Eli Lilly and Company, Indianapolis, Ind.

avidity sufficient to make their serum entirely satisfactory for use in routine blood grouping. In the present experiment the titer of the group B sera against A_2 cells was not determined before the injection was made, since it was decided to use one of the donors in any case. The anti-A_2 titer which is shown by Subject 3, however, indicates that there had been a great increase in activity for this subgroup, and earlier work[2] shows that group B individuals, in fact, often show much greater relative increases in titer to A_2 than to A_1, as well as great increases in avidity for A_2. This is one of the notable advantages of the Witebsky method of producing blood grouping sera, for reagents prepared in other ways often show inferior agglutinating power for the A_2 antigen, especially when it is in combination with the antigen B in the subgroup A_2B.[1] Since many of the errors in grouping which are made in hospitals and in the Armed Forces probably result from a failure to recognize the presence of the antigen A_2, this is a point of considerable importance.

TABLE I. CHANGE IN TITER* OF ISOAGGLUTININS TWO WEEKS AFTER AN INJECTION OF A AND B SUBSTANCE

SUBJECT	GROUP BLOOD	INITIAL TITER		FINAL TITER		
		A_1 CELLS	B CELLS	A_1 CELLS	A_2 CELLS	B CELLS
1	B	128	--	512	128	--
2	B	512	--	8192	512	--
3	B	512	--	2048	1024	--
4	A	--	32	--	--	256
5	A	--	64	--	--	128
6	A	--	64	--	--	256
7	A	--	32	--	--	64
8	A	--	16	--	--	512
9	A	--	8	--	--	16

*Titer equals reciprocal of highest active dilution.

About 300 c.c. of blood were taken from Subject 2 and about 400 c.c. from Subject 8. The amounts of serum recovered from the clotted bloods were about 150 c.c. in each case. These sera seem to keep their activity indefinitely when kept frozen in the deep freeze.

It is interesting to speculate on the possible military applications of Witebsky's methods, in case large amounts of good blood grouping reagents should suddenly be desired in time of war. Suppose we have the problem of grouping 10,000,000 members of the Armed Forces. About twenty tests can be obtained from 1 c.c. of serum, but let us assume that we get only fifteen tests per cubic centimeter. Let us further assume that only one-half of our volunteers respond with the production of satisfactory isoagglutinins and that we obtain an average of 200 ml. of serum per volunteer. (Actual experience indicates that more serum than this can easily be obtained and, furthermore, that the agglutinin titer often remains high enough to enable successive bleedings to be taken at safe intervals. Moreover, many of the sera obtained are so potent that they can be diluted 1:4 or 1:8 with saline before use.) From these very conservative figures we can compute that $2 \times 10{,}000{,}000/(15 \times 200) = 6{,}666$ volunteers of group A and an equal number of group B will be required. This is a perfectly reasonable number of persons to deal with when one considers the large numbers that can be

vaccinated in a short time, and it is altogether probable that the favorable factors mentioned would reduce the number to little more than 1,000 volunteers of each group, with the result that a single organized center could produce enough blood grouping sera to determine the blood groups of the nation's entire Armed Forces. The expense would be largely confined to costs of personnel to give the injections, determine the final titers,* and package the product. Aside from the packaging and distributing costs, the total expenses might be less than $1,000.

SUMMARY

A routine use of the Witebsky method of producing potent grouping sera is described, and some considerations as to its suitability and low cost, in peace and in war, are offered.

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*Actually, experience indicates that one of the important factors in the inferiority of blood grouping materials prepared in large batches is due to erroneous inclusion in a pool of serum[6] of the serum of one or more individuals who have been erroneously grouped, so it is probable that the best use of technical personnel would be to check the blood groups of each volunteer at least twice. Then the sera of all A volunteers and all B volunteers could be pooled, without doing titers. The sera of the subjects who failed to respond would simply dilute the strength of the final pool, probably by not over a factor of 2, which is easily allowable (see text).

A SIMPLIFIED "QUALITATIVE" METHOD FOR HETEROPHILE ANTIBODY DETERMINATION USING CAPILLARY BLOOD AND A WHITE CELL PIPETTE

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SINCE the demonstration of a heterophile antibody in the serum of patients with infectious mononucleosis in 1932[1] many recognized methods for its determination have been published, all of which depend on the use of inactivated serum. An increasing interest in this disease and the recognition of ambulatory and asymptomatic forms[2, 3] suggested that a simplified "qualitative" test might be of value in clinic and survey work.

The purpose of this paper is to describe the technique of such a test, using capillary blood and a white cell pipette, and to report the results obtained with its use.

EXPERIMENTAL CONSIDERATIONS AND PROCEDURES

For purposes of comparison with methods using serum for the determination of sheep cell agglutination, dilutions of whole blood must be considered in terms of the amount of heterophile antibody actually present. Since red cells occupy roughly one-half the volume of whole blood in normal individuals, two times any single dilution of whole blood would be the approximate equivalent of any similar dilution of plasma or serum alone in terms of the amount of heterophile antibody. The term equivalent titer will be used in this paper to express this relationship when whole blood dilutions are compared with serum dilutions. This same relationship should be true of most patients with infectious mononucleosis since this disease is rarely associated with an anemia.

Another factor bearing on the use of unheated whole blood for agglutination tests is the presence of hemolysins against sheep cells in normal human serum and in the serum of patients with infectious mononucleosis. Since actual demonstration of this capacity usually depends on the addition of complement as well as on incubation in a water bath, it was believed that less difficulty would be encountered from hemolysis if these procedures were avoided. With these considerations in mind, an experiment with whole blood was carried out to determine the practical applications of these concepts.

Whole Blood Method.—Serial twofold dilutions of citrated whole blood were made in saline solution in amounts of 1.0 ml. each. The tubes were centrifuged; 0.5 ml. of the supernate in each tube was mixed with an equal amount of 1 per cent suspension of sheep red blood cells and centrifuged at 2,000 r.p.m. for one to two minutes. The tubes were then shaken and the highest final dilution to show visible agglutination was taken as the end point; this was doubled to obtain titers comparable to dilutions of serum alone, and they were recorded as such.

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Standard Method.—The technique of Stuart[4] as modified by the Connecticut State Department of Health Laboratories was used. To twofold serial dilutions of inactivated serum in amounts of 0.5 ml. each, an equal volume of 1 per cent sheep red blood cell suspension was added; the tubes were incubated in a 37° C. water bath for two hours, and placed in the icebox overnight. Readings were made the following morning after shaking, and the highest final serum dilution showing visible agglutination was taken as the titer. Agglutination titers of 1:160 or more were considered as definitely elevated and are referred to as "positive" in this paper.

The results of simultaneous determination of sheep red blood cell agglutination titers by the methods outlined are listed in Table I for ten sera, five of which were from patients with proved infectious mononucleosis. It appears that the equivalent titers obtained by both methods were closely comparable. Furthermore, it should be recorded that no hemolysis of sheep red blood cells was observed in the experiments in which unheated whole blood was employed. These observations appeared to substantiate the theoretic considerations discussed previously and formed the experimental basis for the simplified test.

TABLE I. COMPARATIVE RESULTS OF SHEEP RED BLOOD CELL AGGLUTINATION TESTS WITH WHOLE BLOOD AND WITH INACTIVATED SERUM AS MEASURED IN TEN SAMPLES OF BLOOD

SAMPLE	SHEEP R.B.C. AGGLUTINATION TITER*	
	WHOLE BLOOD METHOD	STANDARD SERUM METHOD
1	20	40
2	20	40
3	40	40
4	40	40
5	80	80
6	160	160
7	640	640
8	640	640
9	640	640
10	1280	1280

*Titer is expressed as reciprocal of serum dilution.

THE SIMPLIFIED "QUALITATIVE" METHOD

1. Capillary blood was drawn up to the 0.5 mark in a standard white cell pipette and diluted to the 11 mark with normal saline.

2. This mixture was placed in a small test tube measuring approximately 7 cm. in length and with a diameter of 0.5 cm., and an equal volume of saline was added by refilling the white cell pipette to the 11 mark.

3. The tube was centrifuged at approximately 2,000 r.p.m. for one to two minutes.

4. The supernatant fluid was drawn up to the 11 mark on the white cell pipette and mixed with an equal volume of 1 per cent sheep red blood cell suspension in another small tube.

5. The test tube was centrifuged as before at 2,000 r.p.m. for one to two minutes and then shaken or struck sharply with the tips of the fingers until the sedimented cells became dislodged from the bottom of the tube. If definite evidence of agglutination was seen after thorough shaking, the test was recorded as "positive"; if no agglutination was visible and the red blood cells had become resuspended, the test was recorded as "negative."

The term positive is used in the same sense as that just described under Standard Method, that is, a titer of sheep red blood cell agglutination considered unequivocally elevated, while the term negative includes all titers occurring within the normal range. The use of these terms appears justified since the equivalent titer used in the simplified test in these experiments actually corresponds to a dilution of serum of 1:160, previously taken as diagnostic in the standard method. It should be mentioned that a dilution equivalent to 1:80 can be obtained merely by drawing blood up to the 1.0 mark on the white cell pipette and proceeding as described.

While the designations positive and negative were always used in reporting results, a rough estimate of the actual titer in terms of serum dilution could be gained by observation of the type of agglutination: (1) High titer serum resulted in a tightly packed mass of agglutinated red blood cells that was dislodged easily from the bottom of the tube but tended to remain in large clumps on shaking; (2) titers in the low "positive" range of 1:160 and 1:320 resulted in more finely dispersed agglutinated particles; and (3) titers in the high normal range of 1:80 suggested agglutination when the tube was first agitated, but rapidly went into suspension.

Sheep red blood cell suspensions were often found suitable without rewashing for as long as five days if stored at icebox temperatures.

COMPARISON OF RESULTS OBTAINED WITH THE SIMPLIFIED AND THE STANDARD METHODS

One hundred twenty "qualitative" tests performed on capillary blood by the simplified method were checked by the standard method with serum obtained on the same day, and the results are listed in Table II. It will be seen that in no instance was there disagreement between the results obtained by the two methods: The results of ninety-four tests which were negative by the simplified method using capillary blood, when checked by the standard method using inactivated serum, showed titers that fell into the normal range. Similarly, all twenty-six positive tests using the simplified method were confirmed by finding significantly elevated sheep red blood cell agglutination titers by the standard method. It should also be noted that the results of the simplified test agreed with those of the standard method even at "borderline" titers of 1:80 and 1:160.

Eighty-four additional tests have been carried out by the simplified method. Of these, ten were found to be positive, and subsequent determination by the standard technique on the serum of seven of them confirmed these results. The remainder of the eighty-four tests were negative and were in agreement with the final diagnosis both from a clinical and a hematologic standpoint but were not checked by a standard quantitative method.

TABLE II. COMPARATIVE RESULTS OF SHEEP RED BLOOD CELL AGGLUTINATION TESTS

	STANDARD METHOD										SIMPLIFIED METHOD	
	NEGATIVE*					POSITIVE†					NEGATIVE	POSITIVE
TITRE‡	<10	10	20	40	80	160	320	640	1280	2560		
Number of tests	20	29	23	15	7	3	3	11	7	2	94	26
Total tests			94					26			94	26

*Negative = Sheep red blood cell agglutination titer within the normal range.

†Positive = Sheep red blood cell agglutination titer within the range diagnostic of infectious mononucleosis, namely, 160 or more.

‡Titer is expressed as reciprocal of serum dilution.

DISCUSSION

The technique of a simplified "qualitative" method of determination of heterophile antibody has been described and the results obtained with this test have been confirmed with a standard quantitative method in 120 instances. The simplified method as used in these experiments was set up at a dilution equivalent to that of a 1:160 dilution of serum. While it has been stated that the presence of agglutination at this titer has been regarded as unequivocal evidence of a significantly elevated titer, it is evident that no precise dividing

point can actually be determined between the normal and the abnormal. It is felt, however, that agglutination at this titer occurs in a negligible number of normal human sera and can usually be accepted as diagnostic of infectious mononucleosis in the absence of recent serum injections. For those desiring a more sensitive test that might indicate early or convalescent cases of infectious mononucleosis, the simplified test is easily adjustable to the equivalent serum dilution of 1:80 as described under Method. Thus at the higher dilution the test can be used as a diagnostic tool and at the lower dilution for survey work during a suspected epidemic.

SUMMARY

1. Theoretic and practical considerations pertaining to the use of whole blood instead of inactivated serum for the determination of sheep red blood cell agglutination have been discussed and investigated.

2. A simplified "qualitative" method of heterophile antibody determination using capillary blood and a white cell pipette has been described.

3. Comparative results of 120 tests determined by the simplified method have been checked using a standard technique and no instance of disagreement found.

4. The simplified test is adaptable either to survey work or to diagnosis.

Appreciation is expressed to Dr. John S. Hathaway, Yale University Health Department, for suggesting the need for such a test as reported and for aid in collecting data, and to Miss Ruth Northey, of that same department, for technical assistance.

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A SIMPLE METHOD FOR THE DETERMINATION OF SALICYLATES IN BLOOD

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THE following is a rapid and simple method for determining salicylates in the blood. It is based on the use of the xanthoproteic reaction.

TECHNIQUE

Mix thoroughly 1 ml. plasma or serum with 9 ml. trichloracetic acid (7 per cent) and let stand fifteen minutes. Filter through a Whatman No. 5 paper (the filtrate must be absolutely clear). Deliver 2.5 ml. of filtrate into a test tube marked at 10 ml. and mix thoroughly with 1 ml. HNO_3 (concentrated). Add distilled water to 5 ml. and mix again. Place in boiling water bath for five minutes. Cool in cold water bath. Add 3 ml. NaOH (60 per cent), mix thoroughly, and cool in cold water bath. Dilute to 10 ml. with water, mix, and read in colorimeter.

RESULTS

The readings by means of the photoelectric colorimeter* with filter No. 511† were plotted against the amounts of sodium salicylates dissolved in normal human plasma‡ (see Table I). Under these experimental conditions the intensity of the yellow color is found to be directly proportional to the concentration;

TABLE I. RECOVERY VALUES OF SODIUM SALICYLATES IN HUMAN PLASMA IN COMPARISON WITH THE THEORETIC READINGS

CONCENTRATION OF SALICYLATE C (MG. %)	COLORIMETER READING R	COLORIMETER READING R	COLORIMETER READING CORRESPONDING TO 8 C
5	39	39	40
10	80	80	80
15	120	120	120
20	161	161	160
25	200	200	200
30	236	240	240
35	273	273	280
40	314	324	320
45	358	353	360
50	404	407	400
55	449	449	440
60	484	478	480
70	566	566	560
80	646	646	640

From the Division of Chemistry, Laboratories of the Mount Sinai Hospital.
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*Klett-Summerson.

†Corning Glass Works.

‡Human sera contain small amounts of phenol derivatives which contribute to the xanthoproteic reaction. Although they do not correspond to more than 3 mg. per cent (except in cases of renal insufficiency, hepatitis, and a few rare conditions[2]), this factor was eliminated by preparing the curve with dilutions of salicylates in human serum.

in the present case, by a factor of 8. Thus R, the colorimeter reading, equals 8 × C, the concentration of salicylate in milligrams per 100 cubic centimeters.

A series of comparative determinations with our technique and that of Coburn[1] showed them to be in good agreement (see Table II).

TABLE II. COMPARISON OF VALUES BETWEEN XANTHOPROTEIC REACTION AND THE TECHNIQUE OF COBURN[1]

NO.	XANTHOPROTEIC	COBURN	DIFFERENCE (MG. %)
1	8.5	9.2	− 0.7
2	37.5	40.0	− 2.5
3	25.5	23.3	+ 2.2
4	15.0	15.0	0
5	7.5	7.9	− 0.4
6	12.0	11.7	+ 0.3
7	15.0	15.0	0
8	6.5	6.8	− 0.3
9	41.5	38.2	+ 3.3
10	15.0	13.5	+ 1.5
11	32.0	29.6	+ 2.4
12	39.0	37.2	+ 1.8
13	47.0	48.0	− 1.0
14	35.0	33.6	+ 1.4
15	32.0	32.0	0
16	37.5	40.6	− 3.1
17	38.5	34.0	+ 4.5
18	41.0	40.7	+ 0.3
19	33.0	30.6	+ 2.4
20	18.0	14.2	+ 3.8
21	55.0	49.0	+ 6.0
22	6.0	5.0	+ 1.0
23	30.0	30.8	− 0.8
24	29.0	32.6	− 3.6
25	11.5	10.0	+ 1.5

A series of standard potassium dichromate solutions may be used in place of the photoelectric colorimeter. The shade of the yellow color of diluted potassium dichromate perfectly matches that of the xanthoproteic reaction, as given by salicylate. These potassium dichromate solutions may be used either in the visual colorimeter or in sealed tubes to be matched directly by eye, as in the evaluation of the icterus index. For this purpose 4.08 Gm. of potassium dichromate are diluted to 1 liter to make a stock solution from which the series of standards is prepared. If the stock solution is diluted ten times, the resulting solution is equivalent to 60 mg. of salicylate. Further dilutions may be prepared for values down to 10 mg. per cent.

SUMMARY

A simple and rapid method for the determination of blood salicylates, based on the use of the xanthoproteic reaction, has been described.

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THE DETERMINATION OF THE HEART RATE IN THE RAT

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SEVERAL investigators have presented data regarding the heart rate in the rat. Woodbury and Hamilton[1] used the closed optical manometer to compare several species and reported an average rate of 420 per minute. A more usual procedure at present is to calculate the rate from the electrocardiographic tracing.[2, 3] This latter method has yielded rates averaging 430 per minute.

In view of the fact that these methods require a certain amount of manipulation of the animal, it appeared important to devise a method which would give absolute data for the rat at rest. If this could be achieved, other methods would then be amenable to interpretation. Such a method would be of still greater value in the experimental approach to cardiovascular disease if it recorded not only the rate of the heart, but gave some indication of the force and character of the beat as well. An electric apparatus was devised to meet these requirements.

APPARATUS

The apparatus consists essentially of a crystal pickup arranged to receive impulses directly from the chest wall. The output is fed into a cathode ray oscilloscope. The pattern of the heart sounds appears on the screen and this is synchronized with the sweep of the time base. The time base is calibrated in cycles per minute so that when one complete heart pattern becomes stationary the heart rate corresponds with the repetition rate of the time base and is read directly from the dial.

Pickup.—For unanesthetized animals the stand and holder shown in Fig. 1 are used. The animal is placed in a cardboard holder of correct size in which an aperture large enough to expose the chest surface has been cut. The holder is placed on the carrier slings with the animal's chest directly over the curved brass chest plate. The carrier slings are racked up or down to bring the plate into contact with the chest. Contact should be firm but not constricting. The heart sounds are conveyed through the chest plate and its connecting arm into the crystal. The crystal used is an astatic cartridge type B2.

Oscilloscope.—Fig. 2 presents the wiring diagram for a three-inch cathode ray oscilloscope with its contained amplifier. This apparatus differs from the commercial varieties only in detail. The amplifier unit is designed to provide high gain with good low frequency response down to about 40 cycles per second, which is suitable for the sound frequencies encountered in this work. The time base is adapted for low sweep rates. The frequency dial is calibrated in cycles per minute, and the potentiometer control should have a linear taper to provide a linear scale. The circuit as designed covers the range of 180 to 720 cycles per minute, although switches conveniently extend this range in two further steps. A synchronization control is available to "lock" the heartbeat with the time base. To do this, the frequency rate must be reduced to approximately one-half that of the heart rate. The first heart cycle is used to trigger the time base and consequently does not appear in full on the screen. The following beat, however, appears in full and the pattern remains steady without the operator having to manipulate the frequency control. When this control is used, the frequency reading is meaningless.

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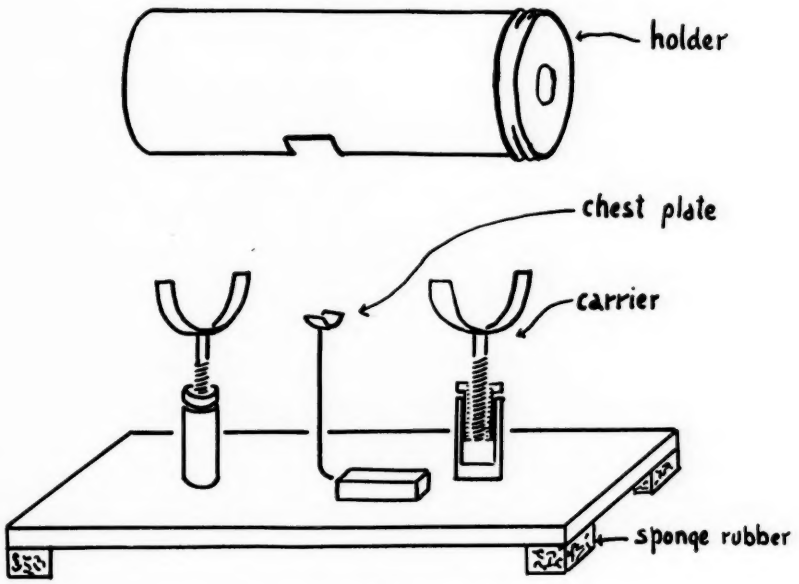
holder
chest plate
carrier
sponge rubber

Fig. 1.

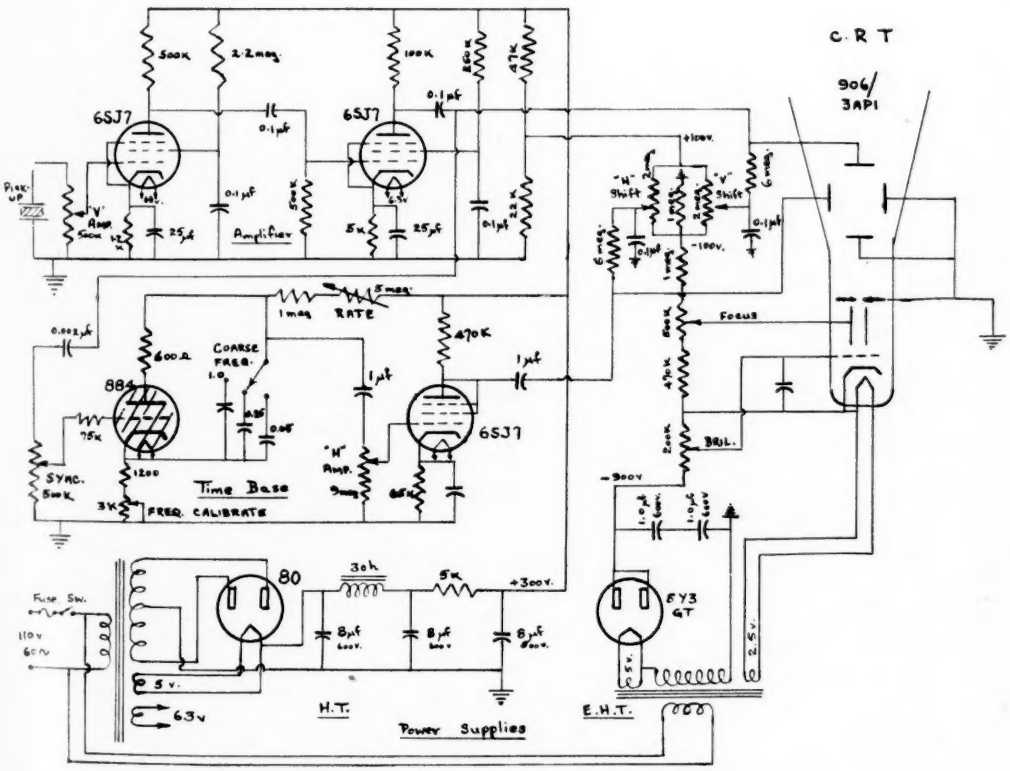
C.R.T
906/
3AP1
6SJ7
6SJ7
6SJ7
884
80
6Y3
GT
Amplifier
RATE
Time Base
FREQ. CALIBRATE
"H" Amp.
"H" Shift
"V" Shift
Focus
BRIL.
H.T.
E.H.T.
Power Supplies
+300v.
-900v.
+100v.
-100v.
110v
60~
5 v.
6.3v

Fig. 2.

EXPERIMENTAL

Data for the heart rate in the unanesthetized rat as well as in animals anesthetized with ether or nembutal are presented in Table I. The normal heart rate of 310 ± 30 in trained unanesthetized animals averaging 150 grams in weight is lower than that previously reported. Nembutal depresses the rate only very slightly, while ether conspicuously increases it.

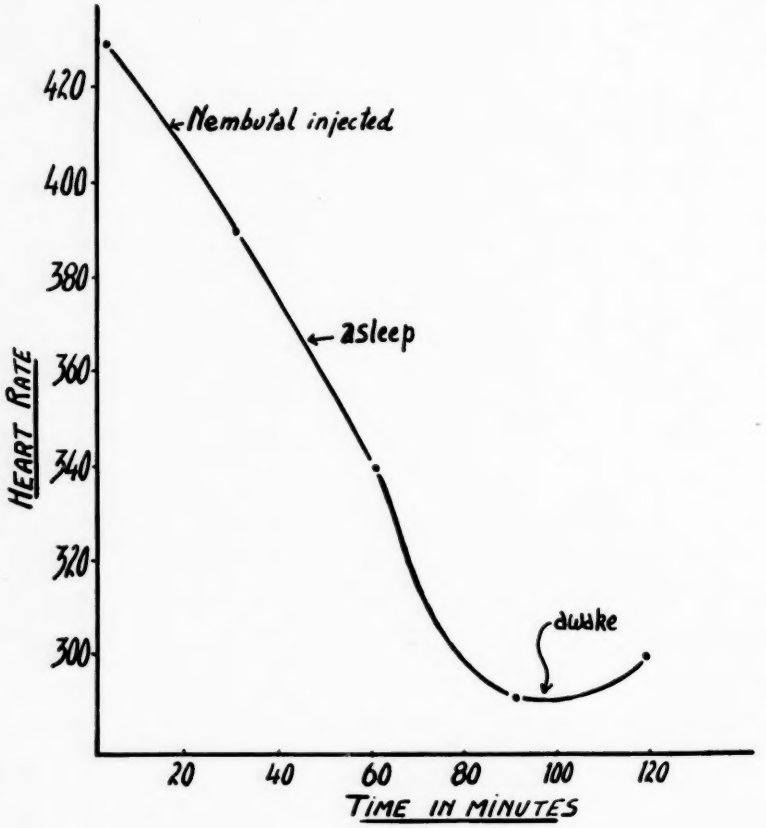

Fig. 3.

TABLE I

GROUP	NUMBER OF ANIMALS	CARDIAC RATE
Normal trained	22	310 ± 30
Normal untrained	10	480 ± 20
Ether		
Preanesthesia (trained)	5	300
Anesthesia	5	460
20 minutes postanesthesia	5	310
Nembutal		
Preanesthesia (partly trained)	6	350
60 minutes after injection	6	310
90 minutes after injection	6	310
120 minutes after injection (awake)	6	310

Subsequent work made it clear that the heart rate was extremely labile. A short stem chest contact was prepared and the rats held manually. The average rate for such manually held rats was 480 beats per minute. Indeed, in several such animals we were able to observe an increase from a starting rate of about 300 to more than 450 beats per minute in the space of thirty seconds. Similar results were obtained with animals in holders if they had not previously been trained. It was found that steady and reproducible results could be obtained by placing the animals in holders for one-half hour a day and determining the rate on the third day. To simplify this we use standard millboard mailing cartons with screw lids as holders. A hole for the tail is bored through the lid and an aperture for the chest contact cut in one side. Large groups can thus be handled since the actual determination of rate after training is a very brief procedure.

For most purposes nembutal given intraperitoneally in small doses is very satisfactory and avoids the training period without apparently affecting the heart rate appreciably. Fig. 3 shows the heart rate curve in an untrained animal following a light dose of nembutal. Nembutal is also advisable when the heart sounds are to be studied. The normal heart sound picture shows two characteristic low frequency patterns, one of greater amplitude than the other. These sounds are close together and occupy about one-half the duration of the cardiac cycle. Murmurs can readily be seen and interpreted.

SUMMARY

1. An apparatus for the determination of the heart rate in the rat has been described.

2. The normal heart rate in the unanesthetized rat averages 300 per minute.

3. The apparatus also makes possible the study of heart sounds in this species.

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Erratum

In the article by Pijper, entitled "The Diffraction Method of Measuring Red Blood Cells," in the July, 1947, issue of the JOURNAL, one of the color plates, Fig. 4, is printed upside down.

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